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Physiological Responses of Cotton Genotypes to Water-Deficit Stress during Reproductive Development

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Physiological Responses of Cotton Genotypes to Water-Deficit Stress
during Reproductive Development

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by

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ABSTRACT

Drought stress is one of the major abiotic factors affecting crop growth and limiting production worldwide. Cotton genotypes vary in drought tolerance, and the effects of drought stress on the anatomy and physiology of cotton leaves and roots have been reported. However, information on physiological and metabolic processes of leaves and flowers of modern cotton cultivars under water-deficit stress during reproductive development is not well elucidated. It was hypothesized that water-deficit stress during squaring and flowering stages would impair stomatal conductance and photosynthetic efficiency of leaves, which consequently would result in osmotic adjustment through accumulation of compatible solutes, increased activity of enzymes, and perturbation of carbohydrates metabolism in leaves and flowers of cotton plants, and differences in drought tolerance among the genotypes would exist. Therefore, field and growth room experiments were conducted to evaluate the effects of water-deficit stress during reproductive development on the physiology and metabolism of leaves and flowers of a diverse range of cotton genotypes. Results indicated that water-deficit stress significantly decreased stomatal conductance of cotton plants. Water-deficit stress during the early squaring stage increased activity of antioxidant enzymes, and decreased photosynthetic efficiency and concentrations of pigments of cotton leaves. Carbohydrate metabolism in cotton flowers and subtending leaves was also compromised by water-deficit conditions, with a shift in the carbohydrate partitioning being promoted by the stress, with subtending leaves and bracts as main sources and pistils as main sinks, mainly for sucrose and soluble sugars. In addition, osmotic adjustment through proline accumulation and changes in osmotic potential is a mechanism used by cotton plants to tolerate drought stress. Leaves are more sensitive to water-deficit stress than ovaries, thus with higher osmotic adjustment. Finally, genotypes varied in

tolerance to drought, with genotypes that showed higher osmotic adjustment in leaves and flowers indicating higher tolerance to drought episodes. Osmotic adjustment through accumulation of compatible solutes could be used as an effective tool for drought-tolerant genotypes in plant biotechnology. However, further research is needed for complete elucidation of osmotic adjustment and carbohydrate metabolism in flower tissues of cotton genotypes under drought conditions during the flower development.

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I. INTRODUCTION

Water is one of the most important factors for crop growth and productivity, and water-deficit stress affects morphological and physiological processes and yield development of plants worldwide (Chaves et al., 2009). More than one third of cultivated areas around the world are supplied with inadequate or lack of water (FAO, 2015). Crop productivity has decreased under water-deficit stress, and current changes in world climatic trends might further exacerbate the severity of the problem with notable water shortage likely to occur in some countries and regions in a near future (FAO, 2015). Currently, cotton (*Gossypium hirsutum* L.) yields in the U.S. have shown vast year-to-year variability (USDA, 2015), and this variability may be related to differences in plant genetics and physiological responses to environmental stresses throughout the season (Robertson, 2001). In the U.S. Cotton Belt, water scarcity is a major deterrent to high yields. This was borne out clearly during the past years in Texas and across the U.S. Cotton Belt.

Water is essential for numerous plant functions, including nutrient transport, chemical and enzymatic reactions, cell expansion, and transpiration (Farooq et al., 2009). Under water-deficit stress, plant anatomy and morphology are altered as well as biochemical and physiological processes consequently affecting plant growth (Farooq et al., 2009; Kramer and Boyer, 1995). In general, a plant is defined as drought stressed when cell water potential and turgor decrease, inhibiting normal metabolic processes (Farooq et al., 2009). The effects of water-deficit stress depend on several factors such as severity and duration of drought as well as the growth stage and genotype of the plant (Kramer and Boyer, 1995). Growth, physiological processes and yield of cotton as affected by drought conditions have been reviewed by Loka et al. (2011). Moreover, the importance of effects of water-deficit stress on reproductive units of

cotton plants has increased due to its high contribution to yield (Loka and Oosterhuis, 2012). However, further studies are still needed for a better understanding on the physiology and metabolism of reproductive units of cotton plants grown under water-deficit conditions. Therefore, a series of experiments were conducted in order to elucidate the effects of water-deficit stress during squaring and flowering stages on physiological and metabolic processes of cotton plants, including reproductive units.

II. LITERATURE REVIEW

Cotton (*Gossypium hirsutum* L.) is the most important crop for fiber production in the U.S. and around the world, and it contributes greatly to food industries for livestock, dairy cattle and poultry (NCCA, 2015). Drought stress is one of the major abiotic factors affecting cotton plants and limiting production worldwide (Osakabe et al., 2014). According to Massacci et al. (2008) approximately one third of growing area around the world has inadequate water supplies. Usually, cotton producers rely on rainfall to supply water throughout the season, as only about 35% of the U.S. cotton production is irrigated (Farahani and Munk, 2012). Improvement in irrigation technology has contributed to more efficient water supplies for crops; however, the high costs involved often limit irrigation. For instance, in places such as Brazil and some African countries, irrigation is not frequently used.

Plant nutrient transport, chemical and enzymatic reactions, cell expansion, and transpiration are processes dependent on water (Farooq et al., 2009). Plant growth, including biochemical and physiological processes, and also plant anatomy and morphology are affected by water deficit (Farooq et al., 2009; Kramer and Boyer, 1995). Plants commonly experience drought-recovery cycles throughout the season and the consequences of these episodic stresses

on crop productivity vary according to plant's ability to avoid cell damage during the stress and to recover after relief of the stress (Munne-Bosch and Penuelas 2003; Blum and Ebercon 1981). The plant's response to water-deficit stress also depends on the severity and duration of the stress as well as the growth stage and genotype of the plant (Kramer and Boyer, 1995).

Plants have evolved mechanisms to cope with temporary water limitations in order to ensure their survival and reproduction. Tolerance to low water potential (the maintenance of plant function at limited water availability and/or the recovery of plant water status and plant function after stress) is one of the mechanisms that may involve osmotic adjustment through accumulation of compatible solutes, such as proline and soluble sugars (Xiong and Zhu, 2002; Bray et al., 2000), but it may also be the result of rigid cell walls or small cells. Drought tolerance can also be associated with the efficient scavenging of reactive oxygen species (ROS) formed as a consequence of disturbed metabolism (Sairam and Saxena, 2000).

Cotton is considered to be a relatively drought tolerant crop since it originates from perennial wild types that grow in areas with scarce precipitation (Lee, 1984). Even though cotton possesses an array of mechanisms to alleviate and survive water-deficit stress (e.g., production of antioxidant enzymes, heat shock proteins, accumulation of osmoytes and osmotic adjustment), physiological and metabolic functions such as photosynthesis, stomatal conductance, respiration, as well as energy production, carbohydrate metabolism and ultimately yield are still compromised under water-deficit stress (Loka and Oosterhuis, 2012). One reason for the yield losses in modern cultivars under water-deficit conditions may be due to the domestication of cotton and its cultivation as an annual crop instead of perennial, since most of the drought tolerant characteristics are associated with the indeterminate type of growth (Quisenberry et al., 1981). Substantial variation in tolerance to drought and heat stresses exists between cotton

species, *Gossypium hirsutum* and *G. barbadense*, and between modern and obsolete genotypes (Saranga et al. 1998; Bibi et al. 2008; Brown and Oosterhuis 2010), but the metabolic reasons for this that could be used to find trait for enhancing drought tolerance have not been completely elucidated.

Most of the research on effects of water-deficit stress in cotton reported in the past has mainly focused on yield and water use (Gutstein, 1969; Yoo et al., 2009), or on the physiology of leaves (Rawson and Constable, 1980; Saranga et al., 2004; Pettigrew, 2004), and consequences on yield (Reddell et al., 1987). Recently, more attention has focused on the effects on the physiology and the metabolism of cotton's reproductive units that ultimately determine yield (Loka and Oosterhuis, 2012). Although all stages of cotton development are sensitive to drought stress, the reproductive phase of flowering and to a lesser extent the boll development period are generally accepted as the most sensitive stages (Loka et al., 2011). In addition, there is evidence that the early stages of square development when meiosis is taking place is also a sensitive stage (Lewis et al., 2000). However, there is very little information on the effects of water-deficit stress on squaring and flowering stages of modern cotton cultivars. Therefore, the knowledge on drought tolerance of modern cotton cultivars during reproductive development is crucial for maintaining production in regions where water supply is limited.

A. EFFECTS OF WATER-DEFICIT STRESS ON MORPHOLOGICAL PROCESSES

Cell growth is the first and one of the most sensitive processes to drought stress due to the decrease in turgor (Taiz and Zeiger, 2010) resulting in decreased leaf area index, leaf size, and stunted plant growth. Additionally, reduced cell turgor and water potential lead to a reduction in leaf expansion and ultimately to lower photosynthetic rates (Ludlow and Muchow, 1990). If the

photosynthetic apparatus is impaired, it results in premature leaf senescence and consequently reduction in yield (Wahid and Rasul, 2005). According to Pace et al. (1999) drought-stressed cotton plants had lower height, leaf area, nodes and dry weights of stems and leaves compared to the control.

Root growth is important for plant development as water and nutrient uptake occur mainly via the roots. Root elongation is affected by water deficit in the soil ultimately limiting crop yield (Bengough et al., 2011). Pace at al. (1999) reported that cotton plants cultivated under drought stress had greater taproot length although the taproot weight was similar to the control, indicating that stressed cotton plants have an increase in root length with the expense of root thickening.

Under severe water deficit, cell elongation of plants may be inhibited by interrupting the water flow from xylem to the surrounding cells. Impaired mitosis and cell expansion result in reduction of root growth, nutrient uptake, plant height, and leaf area as well as plant growth under drought stress (Nonami, 1998; Gunes et al., 2008). Moreover, water-deficit stress affects nutrient supply to the reproductive organs, which inhibits the development of reproductive structures causing fruit abortion (McLaughlin and Boyer, 2004).

B. EFFECTS OF WATER-DEFICIT STRESS ON PHYSIOLOGY

Water-deficit stress affects physiological processes in plants, resulting in alterations in photosynthetic rate, transpiration rate, stomatal conductance, carboxylation efficiency, and water use efficiency in plants (Farooq et al., 2009). Photosynthesis in leaves is reduced under drought stress initially due to stomatal closure which results in lower CO_2 internal concentrations and decreased CO_2 fixation (Flexas et al., 2004). As the stress becomes more severe metabolic

processes such as ribulose bisphosphate (RUBP) synthesis and adenosine triphosphate (ATP) synthesis are also inhibited decreasing photosynthesis rates (Sharkey, 1989; Chaves, 1991; Cornic, 1994).

Pettigrew (2004) observed that cotton leaf photosynthesis increased under drought stress in plants cultivated in the field, while Massaci et al. (2008) indicated that leaf photosynthesis was not affected by the onset of drought whereas photorespiration increased. According to Wullschleger and Oosterhuis (1990) photosynthesis and stomatal conductance of cotton leaves decreased by moderate and severe drought stress while bract photosynthesis and stomatal conductance were not affected by the stress.

Pallas et al. (1967) reported that respiration rates of water-stressed cotton leaves exhibited a biphasial response with respiration decreasing at the onset of stress and then increasing once the stress becomes more severe. Moreover, according to Wullschleger and Oosterhuis (1990) respiration rates of cotton bolls were unaffected under moderate drought stress and decreased when severity of stress increased.

There is a root-to-leaf chemical signal caused by water-deficit stress, namely abscisic acid (ABA) that leads to stomatal closure. Under drought stress, ABA promotes stomata closure reducing transpiration (Taiz and Zeiger, 2010). When water potential in plants is low, ABA also stimulates root growth and inhibits shoot growth (Taiz and Zeiger, 2010). Once the soil water availability is reduced, the amount of ABA in xylem increases and consequently ABA concentration in different parts of the leaf increases as well. As ABA is directly associated with stomatal conductance, it reduces net CO_2 uptake, decreasing photosynthesis.

Under severe water-deficit stress, photosynthesis is also impaired by nonstomatal factors, including a reduction in carboxylation efficiency which leads to excess of absorbed light energy

in photosystem II (PSII). This could damage the photosynthetic apparatus through increased production of chlorophyll triplet if excess energy is not properly dissipated (Long et al., 1994).

Photosystem II is the first complex in the electron transport chain in the photosynthesis process, responsible for oxidation of water and generation of molecular oxygen (Lambers et al., 2008). Efficiency and stability of PSII can be accessed through measurement of maximum quantum yield of PSII (F_v/F_m). Therefore, F_v/F_m is known as an indicator of damage in PSII complexes induced by stress conditions (Rees et al., 1990; Krause and Weis, 1991; Lazar and Naus, 1998). Previous research has established that F_v/F_m is tolerant to water-deficit and high temperature conditions (Pettigrew, 2004; Lawlor and Tezara, 2009; Snider et al., 2013). Snider et al. (2013) documented that PSII heat tolerance was higher when maximal drought stress exposure occurred throughout the growing season for rain fed *G. hirsutum* plants. These results suggest that improved PSII heat tolerance might be related to the acclimation response to water deficit, even though growth room studies demonstrated that increased PSII heat tolerance in drought stressed, relative to control plants, are limited in *G. hirsutum*. To our knowledge, studies evaluating differences in PSII heat tolerance between *G. hirsutum* and *G. barbadense* in response to water-deficit conditions are non-existent.

In addition, research on photosynthetic efficiency of plants under water-deficit stress has been reported to be a useful indicator for tolerance due to its sensitivity to water scarcity conditions (Rong-hua et al., 2006). Chlorophyll fluorescence is a fast, precise and non-destructive measurement, with a positive relationship between the actual quantum yield of PSII and the quantum efficiency of CO_2 fixation (Genty et al., 1989). In cotton plants, chlorophyll fluorescence by means of SPAD measurement has been documented to decrease in plants grown

under water-deficit stress conditions (Ennahli and Earl, 2005; Kitao and Lei, 2007), decreasing photosynthesis rate and sugar production.

Some studies have indicated that water-deficit stress also affects photosynthetic pigments, damaging the photosynthetic apparatus, and decreasing the activities of Calvin cycle enzymes, which are the major causes of reduced yield (Anjum et al., 2003; Fu and Huang, 2001; Monakhova and Chernyadèv, 2002). The photosynthetic pigments are important as they participate on the processes of light energy absorption for further conversion into ATP and nicotinamide adenine dinucleotide phosphate (NADPH) during the light reaction of the photosynthesis process. The pigments commonly found in plants are chlorophyll *a*, chlorophyll *b* and carotenoids. Chlorophyll *a* is the primarily responsible for the green color in plants and carrying out photosynthesis (Taiz and Zeiger, 2010). Chlorophyll *b* helps to increase the absorption band of light to be used in photosynthesis. This pigment aids chlorophyll *a* transferring the absorbed energy to the molecules that carry out photosynthesis (Raven, 1983). Under high light intensity, plants can absorb more light energy than actually used in photosynthesis process. The overexcitation of chlorophyll may result in increased formation of chlorophyll triplet and singlet oxygen. Damage caused by singlet oxygen and its reactive products reduce the efficiency of photosynthesis through photoinhibition. When carotenoid biosynthesis is blocked by addition of inhibitors or mutation and the plant is exposed to ambient light intensity in the presence of O_2, lethal concentrations of singlet oxygen are accumulated. The carotenoids are capable of receiving the triplet excitation energy of chlorophyll and thus help to prevent the formation of singlet oxygen (Malkin and Niyogi, 2000). Several studies have shown reduction in photosynthetic pigments concentration in many crops, such as wheat (*Triticum*

aestivum L.), chickpea (*Cicer arietinum* L.), and sunflower (*Helianthus annuus* L.) under drought stress (Nikolaeva et al., 2010; Mafakheri et al., 2010; Manivannan et al., 2007).

Plant growth depends on the appropriate supply of photosynthetic assimilates. Drought stress significantly decreases photosynthesis in plants, resulting in reduced leaf carbohydrate concentrations due to impairment of acid invertase. Water deficit also affects the tissue reserves because respiration continues to demand substrate (Saini, 1997). Thus, several cellular processes are reduced as photosynthesis is decreased (Boyer and McLaughlin, 2007). Sucrose content of ovary cells is reduced by water-deficit stress as well as glucose due to consumption of starch in ovary tissues. However, these changes are dependent on the delivery and location of the sugars (Mäkela et al., 2005).

Reduced water potentials decrease activity of fructose 2,6-bisphosphate (FBPase) and sucrose phosphate synthase (SPS) (Haupt-Herting and Fock, 2002). The inhibited activity of FBPase and SPS regulates the synthesis of sucrose and starch as well as their partitioning under drought stress. The change in starch and sucrose ratio causes alterations in the inorganic phosphorus (Pi) flux across the chloroplast membrane. Thus, the reduction of Pi in the chloroplasts inhibits ATP synthesis with a great impact on photophosphorylation and photosynthetic carbon reduction (PCR) cycle (Tezara et al., 1999).

Carbohydrate metabolism changes when plants are subjected to water-deficit stress resulting in accumulation of soluble sugar (Chaves, 1991). Expanded cotton leaves export fixed carbon even under low water availability in the cells, which might be related to cell turgor due to osmotic adjustment through accumulation of soluble sugars as an adaptation mechanism to tolerate water-deficit stress (Kadkhodaie et al., 2013; Chaves, 1991).

Modern cotton cultivars vary in tolerance to abiotic stresses, such as drought (Brown et al., 2004). Therefore, maintenance of plant cell turgor for physiological and metabolic processes responsible for plant growth and productivity is an indication of potential drought tolerance of a cultivar (Mir et al., 2012). However, as drought stress becomes more severe, energy and carbon supply by the plants is decreased and demand is increased, consequently reducing growth rates (Chaves, 1991). Water-deficit stress causes a disproportion in carbon partitioning in the plants by limiting production and consumption of photoassimilates (Chaves, 1991). Changes in water potential and carbohydrate metabolism in leaves and fruits are indicators of the degree of severity of water-deficit stress in plants (Fessender and Ehleringer, 2003). Mild drought stress was documented to reduce starch concentration and increase soluble sugars concentration in numerous species (DaMatta et al., 1997; Basu et al., 1999).

Under well-irrigated conditions, cotton plants produce and accumulate assimilates during daylight hours and translocate the reserves to sinks at night (Warner and Burke, 1993). Leaves are the main source of photoassimilates in the plants with translocation to developing bolls at high rates, and subtending leaves contribute with approximately 60% of the total assimilates translocated to fruit under well-watered conditions (Schubert et al., 1986). Under water-deficit conditions, plant growth is impaired and carbohydrates metabolism changes with an increase in sucrose compared with well-watered plants (Timpa et al., 1986). Some studies have showed that plants, such as cotton, soybean (*Glycine max* L.), and pigeonpea (*Cajanus cajan* L.), under drought stress exhibited reduction in starch concentration and increase in hexose sugars in the leaves, indicating that the source-sink relationships are affected by water deficit (Eaton and Ergle, 1948; Liu et al., 2004; Keller and Ludlow, 1993). Research on maize (*Zea mays* L.) indicated that drought stress promoted a higher accumulation of sucrose in the leaves due to a

reduction in sink consumption accompanied by an increase in starch-breakdown enzymes, which reduced starch concentrations in the leaves (Zeeman et al., 2004; Quick et al., 1989). Changes in carbohydrate metabolism in reproductive organs of wheat plants that experience drought episodes cause pollen sterility (Dorion et al., 1996; Saini et al., 1984). In addition, soybean plants grown under water-deficit stress exhibited an alteration in carbohydrates concentration leading to pod absorption (Liu et al., 2004). Moreover, cotton plants subjected to drought stress experience a change in carbohydrate metabolism with an increase in glucose concentrations in leaves and sucrose concentrations in pistils of white flowers (Loka and Oosterhuis, 2013).

C. EFFECTS OF WATER-DEFICIT STRESS ON BIOCHEMICAL PROCESSES

Drought stress also contributes to accumulation of reactive oxygen species (ROS), such as superoxide radicals (O_2^-), hydrogen peroxide (H_2O_2) and hydroxyl radicals (OH). ROS damage plants by oxidation of photosynthetic pigments and destruction of lipids, proteins and nucleic acids (Reddy et al., 2004). In order to eliminate ROS, plants increase activity of antioxidant enzymes, such as superoxide dismutase, catalase, ascorbate peroxidase, and glutathione reductase. The superoxide radicals (O_2^-) are eliminated by superoxide dismutase in a reaction that produces hydrogen peroxide (H_2O_2). H_2O_2 is consumed by conversion to oxygen and water by catalase, or only in water by oxidation of ascorbate. Ascorbate is regenerated by two mechanisms, one through enzymatic reduction of monodehydroascorbate that occurs in plastids or alternatively monodehydroascorbate that is spontaneously dismutated to dehydroascorbate which can react with glutathione (GSH) to produce ascorbate and oxidized glutathione (GSSG) in a reaction catalyzed by dehydroascorbate reductase. The GSSG is reduced by glutathione reductase requiring the consumption of NADPH. The singlet oxygen and hydroxyl ions are removed in the glutathione

pathway. Damage caused by both singlet oxygen and hydroxyl ions is also reduced by non-enzymatic antioxidants, vitamin E and carotenoids (Bray et al., 2000). Mahan and Wanjura (2005) have reported an increase in ascorbate peroxidase activity in cotton plants cultivated under drought stress whereas glutathione metabolism and levels of malondialdehyde (MDA) were not altered by the stress. However, research on antioxidant activity in cotton plants subjected to drought stress is still debatable and not well understood. Some studies indicated an increase in APX activity, while activities of SOD or CAT remained unaltered under drought stress (Ratnayaka et al., 2003), and studies reported that CAT activity was unaffected by mild water-deficit stress and increased activity in plants under severe stress, while SOD activity was higher only in plants under mild stress (Deeba et al., 2012). Activity of antioxidant in plants such as maize (Moussa and Abdel-Aziz, 2008), canola (*Brassica napus* L.) (Mirzaee et al., 2013), quinoa (*Chenopodium quinoa* L.) (Fghire et al., 2013), and potato (*Solanum tuberosum* L.) (Pilon et al., 2014) grown under water-deficit stress has been documented.

Under drought stress, in addition to the production of antioxidants, osmotic adjustment occurs in plant cells through accumulation of compatible solutes in the cytosol (Xiong and Zhu, 2002). The term "compatible solutes" includes amino acids and their derivates, betaine, polyamines, proteins, soluble carbohydrates and polyols. All these compatible solute are highly soluble and do not interfere with cell metabolism even at high concentrations (Bray et al., 2000). Proline is one of the most common compatible solutes in plants under drought stress (Bray et al., 2000). Proline concentrations are maintained by combining the synthesis and catabolism of the amino acid (Bray et al., 2000). Proline accumulation may represent a regulatory mechanism of water loss by reducing the cell water potential (Fumis et al., 2002); however, it also serves as a biochemical marker of metabolic changes caused by stress (Lima et al., 2004). In most plants,

osmotic adjustment has a role of decreasing the osmotic potential of cells, therefore increasing the gradient for water flux inside the cell to maintain cell turgor and growth (Fumis et al., 2002; Nikolaeva et al., 2010; Mafakheri et al., 2010). Maintenance of cell turgor contributes to continued physiological processes, such as stomatal conductance and photosynthesis (Blum, 1996). Cotton has been reported to have the ability to osmotically adjust and maintain a higher leaf turgor potential (ψ_t) (Oosterhuis and Wullscheleger, 1987; Turner et al., 1986; Nepomuceno et al., 1998).

Osmotic adjustment is variable in species according to factors, such as genotype and organ type and age (Morgan, 1984). In cotton plants, higher osmotic adjustment was found in roots than leaves in response to water-deficit conditions (Oosterhuis and Wullschleger, 1987). Osmotic adjustment as a response of drought stress in different crops has also been studied (Borgo et al., 2015; Marechaux et al., 2015; Jamal et al., 2015); however, information on osmotic adjustment in modern cotton cultivars under water-deficit stress is still lacking.

D. EFFECTS OF WATER-DEFICIT STRESS ON YIELD

Drought effects on plant development vary for different species resulting nearly always in yield losses (Mahajan and Tuteja, 2005). After seed germination and seedling establishment, the reproductive development is the most sensitive stage to water-deficit stress in most crops (Saini, 1997). In cotton, the sensitivity to drought stress during flowering and boll development has been well established (Constable and Hearn, 1981; Cull et al., 1981a,b; Turner et al., 1986). The amount of water utilized by cotton plants is related to the efficacy of physiological processes responsible for crop growth and yield (Deeba et al., 2012). Therefore, final cotton yield decreases due to lower photosynthetic efficiency in plants grown under drought stress. In cereal

crops, the most sensitive periods to drought stress are grain filling, accumulation of reserve nutrients and carbohydrates in the developing and maturing grain (Yang and Zhang, 2006).

Research on cereal crops has indicated that water stress at meiosis and early grain formation has detrimental effects on yield due to the sensitivity of floral initiation and pre-meiotic differentiation of the flower to water stress (Winkel et al., 1997; Saini, 1997). The duration of stress is as important as the severity to affect crop yield. Drought stress at floral initiation, pollination, or seed development of cotton causes a decrease in number of seeds, affecting yield. Also, lint yield is reduced by decrease in boll production, due to a reduction in flowering sites and increased boll abscission when plants are exposed to extreme drought during the reproductive development (Turner et al., 1986; Gerik et al., 1996; Pettigrew, 2004). Irrigation rate and form of application affect boll development and retention (Ritchie et al., 2009; Whitaker et al., 2008), reducing dry matter, number and weight of bolls, and lint yield (reviewed by Loka et al., 2011). According to Pettigrew (2004) drought stress affected the vertical and horizontal distribution of bolls in cotton. Plants cultivated under drought stress produced more bolls at the first sympodial fruiting position and fewer bolls above main-stem node 11 compared to the control, resulting in reduction in lint yield due to loss of these fruiting positions as well as the decrease in lint per seed.

Cotton plants exposed to drought stress shortly after flowering have the fiber length significantly decreased. Fiber growth is a process dependent on turgor and carbohydrates supply in the plant (Dhindsa et al., 1975). As drought stress negatively affects plant water relations and photosynthesis, fiber extension is negatively affected (Dhindsa et al., 1975). Lint yield is related not only to fiber quality but also to number of fibers per seed and number of seeds per unit area (Lewis et al., 2000). Plant water availability is strongly correlated to fiber and seed development,

which requires high water uptake to maintain seed growth (Rabadia et al., 1999). Moreover, plants grown under drought stress conditions exhibit an increase in number of unfertilized ovules, decreasing the final yield (Saranga et al., 1998).

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III. PHYSIOLOGICAL RESPONSE OF COTTON CULTIVARS UNDER WATER-DEFICIT STRESS DURING THE SQUARING STAGE.

ABSTRACT

Numerous studies with several species have reported that photosynthetic efficiency of plants, as well as increased activity of antioxidant enzymes, are useful indicators of drought tolerance. However, information on antioxidant response of cotton plants under water-deficit stress is still not well elucidated. Additionally, the effect of drought stress on photosynthetic efficiency of modern cotton cultivars during early reproductive development is not completely understood. Therefore, the objectives of this study were to evaluate changes in the photosynthetic efficiency, concentrations of photosynthetic pigments and activity of enzymes in cotton plants caused by water-deficit stress during the early squaring stage, and identify differences in physiological responses and tolerance to drought among the modern cultivars. Field experiments were conducted in Marianna, AR and Lubbock, TX. Three and one cotton cultivars were planted in Marianna and Lubbock, respectively. Plants were well-watered until the appearance of floral buds, at which time water was withheld for fourteen days. After seven and fourteen days of water-deficit stress, stomatal conductance and photosynthetic efficiency were measured and samples were collected from main-stem leaves from the fourth node below the apical meristem for determination of concentrations of pigments and activity of enzymes. Results indicated that activity of antioxidant enzymes was significantly increased by water-deficit stress during the early squaring stage. As the water-deficit stress became more severe, photosynthetic efficiency and concentrations of pigments were decreased. High accumulation of antioxidant enzymes and carotenoids in water-stressed plants appeared to contribute to scavenger reactive oxygen species.

The cultivar ST5288 exhibited the higher tolerance to water-deficit conditions among the cultivars due to higher photosynthetic efficiency, concentration of pigments and activity of enzymes in stressed plants. Further research is needed to identify the level of impairment of water-deficit stress during the early reproductive stage on physiological processes of cotton plants.

INTRODUCTION

Cotton yield can be compromised by water-deficit stress due to impairment of physiological and metabolic functions such as photosynthesis and stomatal conductance, as well as energy production and carbohydrate metabolism, leading to reduction in plant growth and fruit (boll) abscission (reviewed by Loka et al., 2011). The effects of water-deficit stress in crops vary with the severity and duration of the stress, plant growth stage and genotype, as well as the interaction between these factors (Kramer and Boyer, 1995). Recently, cotton yields in the U.S. have shown great variability across the years (USDA, 2015), and this year-to-year variability might be attributed to differences in plant genetics, and physiological responses to environmental stresses throughout the season (Robertson, 2001).

Upland cotton (*Gossypium hirsutum* L.) possesses an array of mechanisms to alleviate and survive water stress (e.g., increased activity of antioxidants, heat shock proteins, accumulation of osmolytes and osmotic adjustment); however, due to domestication and cultivation as annual crop, modern cotton cultivars differ in their ability and level of tolerance to a water-deficit period (Quisenberry et al., 1981; Loka and Oosterhuis, 2012).

Stomatal closure and increased mesophyll resistance occur shortly after the onset of water-deficit stress, decreasing CO_2 absorption used in the photosynthesis process (Flexas et al.,

2004). Under severe water-deficit stress, photosynthesis is also impaired by nonstomatal factors, including a reduction in carboxylation efficiency which leads to excess of absorbed light energy in photosystem II (PSII). This could result in damage in the photosynthetic apparatus through increased production of chlorophyll triplet if excess energy cannot be properly dissipated (Long et al., 1994).

Studies on photosynthetic efficiency of plants under water-deficit stress have been reported to be a useful indicator for tolerance due to its sensitivity to water scarcity conditions (Rong-hua et al., 2006). Chlorophyll fluorescence is a fast, precise and non-destructive measurement, with a positive relationship between the actual quantum yield of PSII and the quantum efficiency of CO_2 fixation (Genty et al., 1989). In cotton plants, chlorophyll fluorescence has been documented to decrease in plants grown under water-deficit stress conditions (Ennahli and Earl, 2005; Kitao and Lei, 2007), decreasing photosynthesis rate and sugar production.

In addition to chlorophyll fluorescence, photosynthetic pigments are affected by low water availability. The photosynthetic pigments are important as they participate on the processes to absorb light energy for further conversion into adenosine triphosphate (ATP) and nicotinamide adenine dinucleotide phosphate (NADPH) during light reaction of the photosynthesis process. The pigments commonly found in plants are chlorophyll *a*, chlorophyll *b* and carotenoids. Chlorophyll *a* is the main pigment absorbing energy for the light reaction of photosynthesis and also responsible for the green color in plants (Taiz and Zeiger, 2010). Chlorophyll *b* helps to increase the absorption band of light to be used in photosynthesis. Under high light intensity, plants can absorb more light energy than actually used in photosynthesis process. The overexcitation of chlorophyll may result in increased formation of reactive oxygen

species (ROS) such as chlorophyll triplet and singlet oxygen. Damage caused by singlet oxygen and its reactive products reduce the efficiency of photosynthesis through photoinhibition. Carotenoids are capable to receive the triplet excitation energy of chlorophyll and thus help to prevent the formation of ROS (Malkin and Niyogi, 2000). However, when carotenoid biosynthesis is blocked by addition of inhibitors or mutation and the plant is exposed to ambient light intensity in presence of O_2, lethal concentrations of ROS are accumulated. Studies have shown degradation in photosynthetic pigments concentration in several crops grown under drought conditions, such as cotton, potato (*Solanum tuberosum* L.), wheat (*Triticum aestivum* L.), chickpea (*Cicer arietinum* L.), and sunflower (*Helianthus annuus* L.) (Shah et al., 2011; Pilon et al., 2014; Nikolaeva et al., 2010; Mafakheri et al., 2010; Manivannan et al., 2007).

Overproduction of reactive oxygen species (ROS) also damage plant cells irreversibly by degradation of lipids, proteins and nucleic acids (Reddy et al., 2004). Antioxidant enzymes, such as superoxide dismutase (SOD), catalase (CAT), and ascorbate peroxidase (APX) increase their activity to control the balance between production and scavenging ROS. Reactive oxygen species are eliminated by SOD through a reaction that produces hydrogen peroxide (H_2O_2). Then, the H_2O_2 is converted into oxygen and water by CAT or into water by APX. Research has been reported on antioxidant activity of plants under water-deficit stress, such as maize (*Zea mays* L.) (Moussa and Abdel-Aziz, 2008), canola (*Brassica napus* L.) (Mirzaee et al., 2013), quinoa (*Chenopodium quinoa* L.) (Fghire et al., 2013), and potato (Pilon et al., 2014). However, studies on antioxidant responses of cotton plants under drought conditions are still controversial and not well elucidated, with studies indicating increase in APX activity and no alteration in activities of SOD or CAT under drought stress (Ratnayaka et al., 2003) and studies reporting higher CAT activity in plants under severe stress and no changes in plants under mild stress,

while SOD was higher only in plants under mild stress and similar levels of SOD in plants under severe stress and control (Deeba et al., 2012).

Throughout cotton development, the reproductive phase of flowering is generally accepted as the most sensitive stage (Loka et al., 2011). In addition, there is evidence that the early stage of square (floral bud) development when meiosis is taking place is also a sensitive stage (Lewis et al., 2000). However, there is very little information on the physiological responses of cotton plants that experience water-deficit stress during the early squaring stage. Therefore, we hypothesize that activity of enzymes will increase and photosynthetic efficiency will be impaired as cotton plants experience water-deficit stress during the early squaring stage and that variation in drought tolerance will exist among modern cultivars being utilized in the U.S. production.

The objectives of this study were to evaluate changes in photosynthetic efficiency, concentrations of photosynthetic pigments and activity of enzymes of cotton plants caused by water-deficit stress during the early squaring stage, and identify differences in physiological responses and tolerance to drought among modern cotton cultivars.

MATERIALS AND METHODS

Field experiments were conducted at the Quaker Avenue Research Farm of Texas Tech University in Lubbock, TX (N 33°59’93”, W 101°90’72”) and at the Lon Mann Cotton Research Station of the University of Arkansas in Marianna, AR (N 34°43’50”, W 90°45’34”) in 2012 and 2013, respectively. Seeds of one modern cotton cultivar, ST 5288B2F (Stoneville, Bayer CropScience, Lubbock TX) were sown on May 23, 2012 (Lubbock, TX) and three, DP 0912 B2RF (Delta and Pine Land, Monsanto Company, St. Louis MO), PHY 499 WRF (PhytoGen,

Dow AgroSciences, Indianapolis IN), and ST 5288B2F (Stoneville, Bayer CropScience, Lubbock TX) on May 8, 2013 (Marianna, AR) at a 0.96 m inter-row spacing and at a rate of 11 seeds m^{-1} row. A total of 10 and 25 plots were eight and four rows wide at Lubbock and Marianna, respectively, and 15.2 m long. The soil at Lubbock is mapped as an Amarillo-Acuff sandy clay loam (Fine-loamy, mixed, superactive, thermic Aridic Paleustalfs), while the soil at Marianna is mapped as a Memphis silt loam (fine-silty, mixed, active, thermic Typic Hapludalfs). To further characterize site conditions, the average weekly maximum and minimum temperatures, and precipitation of each location throughout the season are presented in Appendix. Fertilization was performed according to soil tests prior to planting and recommended rates for cotton. Herbicide and pesticide applications were also applied according to Texas A&M AgriLife Extension recommendations at Lubbock and University of Arkansas Cooperative Extension Service recommendations at Marianna. Mepiquat chloride was applied as needed to control vegetative growth and all plots received identical applications. Irrigation was supplied as necessary using a subsurface drip system at Lubbock and a furrow system at Marianna according until the appearance of floral buds (squaring stage). When plants reached the pinhead square stage, water was withheld from the water-stress treatment for fourteen days at both locations.

For the Lubbock location, field measurements of stomatal conductance were performed seven and fourteen days after the onset of the stress and samples for laboratory determinations of activities of antioxidant enzymes (SOD, CAT, and APX) were taken fourteen days after the onset of the stress. For Marianna, field measurements of stomatal conductance and chlorophyll *a* fluorescence, and samples for laboratory determinations of concentrations of pigments (chlorophyll *a*, chlorophyll *b*, and carotenoids) were taken seven and fourteen days after the onset of the stress. Both field and laboratory measurements were performed in fully-expanded

main-stem leaves from the fourth node below the apical meristem from the four and two middle rows of each plot at Lubbock and Marianna, respectively.

Stomatal conductance

Stomatal conductance was measured between 11:00 and 14:00 h in five leaves per plot and two readings per leaf (which were averaged) due to the small surface area of the cuvette (6.35 mm^2) using a steady-state leaf porometer (SC-1 Leaf Porometer, Decagon, Pullman, WA) on the abaxial surface of fully expanded main-stem leaf on the fourth node below the apical meristem. The results were expressed as mmol H_2O $m^{-2}s^{-1}$.

Chlorophyll a fluorescence

Actual quantum yield of electron transport through photosystem II (Φ_{PSII}) was measured *in situ* in five leaves per plot under natural field irradiance between 12:00 and 14:00 h using a portable fluorometer Model OS1-FL (Opti-Sciences, Hudson, NH). Steady-state fluorescence prior to a saturation pulse was measured to obtain F_t, followed by a induction of maximum fluorescence on the adaxial surface of the leaves with a saturating white light pulse for 0.95 s for estimation of F'_m when all reactions centers are closed due to infinite light intensity. Φ_{PSII} was obtained according to the equation $\Phi_{PSII} = (F'_m - F_t) / F'_m$ (Maxwell and Johnson, 2000). Electron transport rate (ETR) through photosystem II was obtained by calculation according to the equation ETR = Φ_{PSII} x PAR x 0.5 x 0.84, where PAR is the absorbed light (in µmol photon $m^{-2}s^{-1}$) at the leaf surface, 0.5 is a factor on the partitioning of energy between PSII and PSI and 0.84 is a common leaf absorbance coefficient for C_3 plants.

Pigments concentration

Two leaf discs (10 mm diameter) were collected from five leaves of each plot, placed in vials filled with 1.5 mL dimethylformamide and incubated at ambient temperature (25°C) for 48 h for pigments extraction. After the incubation period, the samples were read in a UV-visible spectrophotometer (UV-1700 PharmaSpec, Shimadzu, Kyoto, Japan) at wavelengths of 480, 646.8, and 663.8 nm for carotenoids, chlorophyll *a* and chlorophyll *b* concentrations, respectively, according to calculations described by Inskeep and Bloom (1985).

Activities of enzymes

The activity of the enzymes superoxide dismutase (SOD), catalase (CAT), and ascorbate peroxidase (APX) were measured in fully expanded main-stem leaf on the fourth node below the apical meristem from the two middle rows of each plot only in Lubbock.

Superoxidase dismutase (SOD; EC 1.15.1.1)

Activity of the enzyme SOD was performed by measuring the enzyme's ability to inhibit photochemical reduction of nitrobluetetrazolium (NBT) according to Giannopolitis and Ries (1977). The activity was determined by adding 50 µL of crude extract into a solution of 13 mM methionine, 75 µM NBT 100 nM EDTA and 2 µM riboflavin in 3 mL of 50 mM potassium phosphate buffer at pH 7.8 in a plate with wells. Reaction started by illuminating the plate with fluorescent light (15 W) at 25°C. After 5 min of incubation, catalysis is terminated by interrupting light. The blue dye formed by photochemical reduction of NBT was determined by absorbance in a UV-visible spectrophotometer (UV-1700 PharmaSpec, Shimadzu, Kyoto, Japan) at a wavelength of 560 nm. The wells on the plate considered as blanks were added with the

same reagents, but they were covered with aluminum foil during incubation in order to avoid photochemical reaction. One unit of SOD was defined as the amount of enzyme required to inhibit 50% of the reduction of NBT. The percentage of the inhibition obtained, the sample volume and the protein concentration (μg μL^{-1}) were considered in the calculations for the enzyme specific activity. Results were expressed as unit of SOD per gram of fresh weight (unit g^{-1} FW).

Catalase (CAT; EC 1.11.1.6)

Catalase activity was measured by monitoring the variation in absorption of hydrogen peroxide (H_2O_2) using a UV-visible spectrophotometer (UV-1700 PharmaSpec, Shimadzu, Kyoto, Japan) at a wavelength of 240 nm, according to Peixoto et al. (1999). For the test, 50 μL of the crude extract were added to 950 μL of potassium phosphate buffer 50 nM at pH 7.0 supplemented with H_2O_2 at final concentration of 12.5 mM. Absorption variation was calculated in an interval of 60 seconds and the CAT activity was calculated using a molar extinction coefficient of 39.4 mM cm^{-1}. The protein concentration (μg μL^{-1}) was also considered in the calculations. Results were expressed as μmol min^{-1} mg^{-1} protein.

Ascorbate peroxidase (APX; EC 1.11.1.11)

For determination of APX activity, initially a solution containing 100 μL of crude extract and 2.9 mL of potassium phosphate 50 mM at pH 6.0 was prepared. Then, ascorbate and hydrogen peroxide at final concentration of 0.8 and 1 mM, respectively, were added to the initial solution. Activity of APX was determining by measuring the negative variation of H_2O_2 absorption in a UV-visible spectrophotometer (UV-1700 PharmaSpec, Shimadzu, Kyoto, Japan)

at a wavelength of 290 nm, according to Koshiba (1993). A molar extinction coefficient of 2.8 mM cm^{-1} was used for the calculations. Results were expressed as µmol min^{-1} mg^{-1} protein.

Statistical analysis

The experiments were arranged in a strip plot design with water treatments running across all blocks in strips at Lubbock and a strip split plot design with water treatments as the main unit running across all blocks in strips in a randomized complete block design and the cultivars were randomly assigned in the sub unit for each whole plot in each block at Marianna with five replications. The treatments water regime and cultivar were considered as fixed effect. The blocks and the block x treatments interaction were considered as random effect. Tukey's test (α=0.05) was used to separate treatment combination mean performance using JMP Pro 11 (SAS Institute, Cary, NC). When interaction between the factors was observed for a variable, a graph was plotted. Otherwise, the differences between the main factors were indicated in the table.

RESULTS AND DISCUSSION

Stomatal Conductance

Leaf stomatal conductance of cotton plants from the two locations was significantly reduced by drought stress (Fig. 1). At Marianna, stomatal conductance rates of water-stressed plants were approximately 50% and 36% lower than the control after seven and fourteen days of stress, respectively (Fig. 1A). Stomatal conductance was not significantly different among the cultivars within the same water regime and sample date. At Lubbock, leaf stomatal conductance of water-stressed plants was 30% lower than the control after fourteen days of stress (Fig. 1B). Both locations showed increase in stomatal conductance over time in all instances (Fig. 1). As

water is one of the most important factors controlling plant growth and development (Hsiao, 1973), physiological processes in the plant, such as photosynthetic capacity and stomatal activity, are impaired under low water availability in the soil (Osakabe et al., 2014). In accordance with this study, research has reported lower stomatal conductance rates in plants cultivated under low water availability in the soil leading to reduction of plant growth and number of reproductive units (Chaves et al., 2002; Mao et al., 2014).

Photosynthetic Efficiency

Stomatal closure leads to reduction in CO_2 assimilation, consequently reducing photosynthesis. Photosynthetic efficiency of plants can be accessed by means of chlorophyll fluorescence measurement. Light energy absorbed by the pigments in the chloroplast can be directed to be used in the photosynthesis, with excess energy being dissipated as heat, or it can be re-emitted as light, that is denominated as chlorophyll fluorescence (Maxwell and Johnson, 2000). Due to a competition between these processes, the chlorophyll fluorescence measurement is used as indication of changes in the efficiency of photochemistry and heat dissipation (Maxwell and Johnson, 2000). In our study, quantum yield of PSII (Φ_{PSII}) was significantly affected by cultivar, sample date and the interaction water regime x sample date (Table 1). The cultivar DP0912 showed the highest Φ_{PSII} followed by ST5288 and lastly PHY499 with the lowest Φ_{PSII}. Quantum yield of PSII was also reduced after fourteen days of stress compared with the first sample date, regardless of the cultivars and water regimes (Table 1 and Fig. 2). Electron transport rate (ETR) was also significantly affected by sample date, with lower rates after fourteen days of stress, regardless of the cultivar or water regime (Table 1). Measurements of the proportion of the light absorbed by chlorophyll associated with PSII and the ETR in the

light reaction are indication of overall photosynthesis. Thus, results of this research suggested that water-deficit stress during the squaring stage did not impair photosynthetic efficiency of the cotton cultivars studied, even with lower stomatal conductance rates found in all cultivars under water-deficit stress regardless of the sample date. Li et al. (2012) found that cotton plants have photosynthetic efficiency impaired by drought stress during the flowering stage, with reduction in quantum yield of PSII and ETR, as well as concentrations of chlorophylls. Studies on soybean, wheat and sorghum also showed reduction in the photosynthetic efficiency of plants grown under drought stress (Mutava et al. 2015; Loggini et al., 1999; Jagtap et al. 1998).

Concentrations of Pigments

In addition to the quantum yield of PSII and electron transport rate, photosynthetic pigments, such as chlorophylls *a* and *b*, and carotenoids are essential for maintenance of the photosynthesis process at high rates. Chlorophylls *a* and *b* were significantly affected by cultivar, water regime and sample date (Table 1). Chlorophyll *a* concentration was also significantly affected by the interaction water regime x sample date (Table 1). Concentrations of chlorophylls *a* and *b* varied among the cultivars, with ST5288 and PHY499 showing higher concentrations of these pigments than DP0912. Low water availability in the cells causes degradation of pigments. The concentrations of chlorophylls *a* and *b* were lower in plants grown under water-deficit stress compared with the well-watered control. Concentrations of these pigments were also reduced after fourteen days of stress in relation to seven days of stress (Table 1). Regardless of cultivar, chlorophyll *a* concentration was decreased by water-deficit stress both after seven and fourteen days after stress (Fig. 3). The reduction in the photosynthetic pigments might impair the photosynthetic process due to lower light harvesting efficiency by the leaves, therefore resulting

in reduced plant growth and productivity. Accordingly to our results, Li et al. (2012) found that concentrations of chlorophylls *a* and *b* in cotton plants were reduced by drought stress over time throughout the season, which included the squaring and flowering stages. Even with lower concentration of the pigments in plants under water-deficit stress, the quantum yield of PSII and electron transport rate were maintained to similar rates found in well-watered plants, which might indicate that the cotton cultivars studied are able to maintain photosynthesis process with lower concentration of photosynthetic pigments present in the cells.

Carotenoids concentration was significantly affected by cultivar, water regime and interaction cultivar x sample date (Table 1). Carotenoids concentration was lower in cells of plants grown under water-deficit stress compared to the well-watered control, regardless of cultivars. After seven days of stress, ST5288 showed the highest carotenoids concentration followed by DP0912 and PHY499, while after fourteen days of stress, ST5288 and PHY499 had higher concentrations than DP0912 (Fig. 4). Carotenoids work not only as an accessory pigment harvesting light for the photosynthesis process but also as an effective non-enzymatic antioxidant in defense against ROS which causes damage in cells. Carotenoids main role as antioxidant is in deletion of chlorophyll triplets produced during photosynthesis, restricting the production of ROS and therefore protecting the cells from oxidative damage (Malkin and Niyogi, 2000). Our results indicate that concentrations of carotenoids are not increased by the stress, suggesting that the cotton cultivars studied in this research do not make use of this mechanism to tolerate water-deficit stress periods.

Antioxidant Profile

Enzymes play a role in cell defense by detoxification the overproduction of ROS, maintaining the balance between formation and removal of ROS in the cells (Reddy et al., 2004). Research on increased enzymatic activity to control rate of ROS in cells has been reported for several crops grown under drought stress, such as maize, canola, quinoa, and potato (Moussa and Abdel-Aziz, 2008; Mirzaee et al., 2013; Fghire et al., 2013; Pilon et al., 2014). In cotton, contrasting results have been found for enzymatic activity of plants subjected to water-deficit conditions. Studies indicated increase in APX under drought and no alteration in SOD or CAT (Ratnayaka et al., 2013), while other studies reported higher CAT activity in plants under severe stress and no changes in plants under mild stress, while SOD was higher only in plants under mild stress and similar levels of SOD in plants under severe stress and control (Deeba et al., 2012). In our study, significant increase in activity of the enzymes SOD, CAT, and APX was detected in plants grown under water-deficit stress (Fig. 5). Water-stressed plants showed a 4-fold and 10-fold increase in SOD and CAT activity, respectively, compared with the control plants (Fig. 5A and B). Ascorbate peroxidase activity was approximately 57% higher in the plants grown under water-deficit stress (Fig. 5C). Improved tolerance to cell damage caused by ROS is observed in plants with higher antioxidant activity. These results suggested that activation of antioxidant enzymes is involved in the mechanism controlling overproduction of ROS and maintaining a balance between production and scavenger of ROS in the cells of the cultivar ST5288.

CONCLUSIONS

Water-deficit stress during the early squaring stage increased activity of antioxidant enzymes, and as the stress became more severe, photosynthetic efficiency and concentrations of pigments were also decreased by water-deficit conditions. High accumulation of antioxidant enzymes and carotenoids in water-stressed plants appeared to contribute to scavenger reactive oxygen species. ST5288 exhibited the most improved tolerance to water-deficit stress among the cultivars due to higher photosynthetic efficiency, concentration of pigments and activity of enzymes in stressed plants.

FIGURES AND TABLES

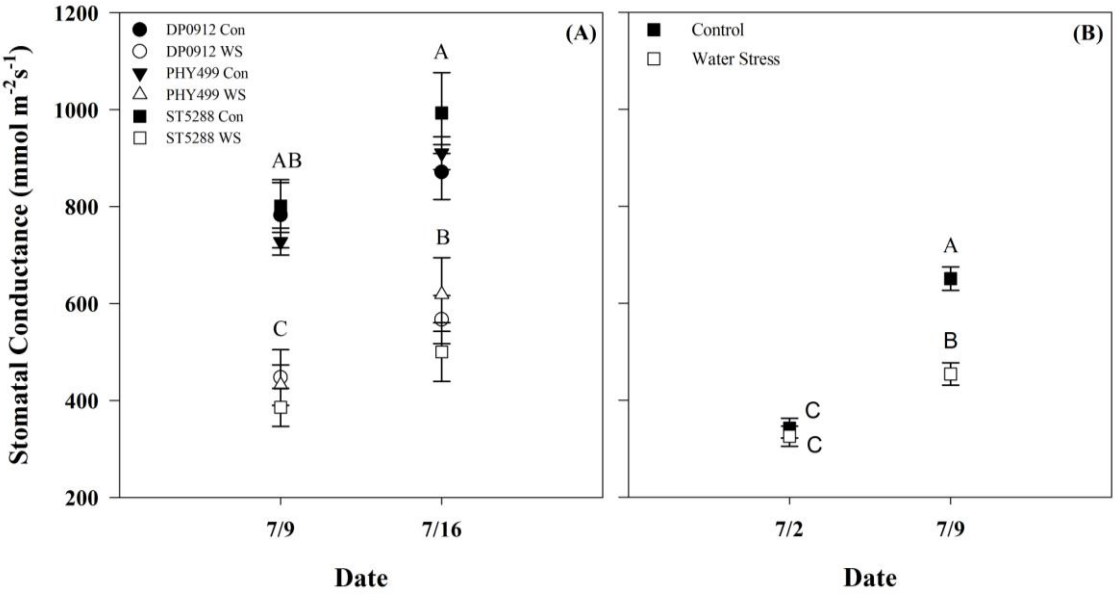

Figure 1. Stomatal conductance (mmol H_2O $m^{-2}s^{-1}$) of three *Gossypium hirsutum* cultivars (DP 0912 B2RF, PHY 499 WRF, and ST 5288B2F) under two water regimes on July 9, and July 16, 2013 in Marianna, AR (A) and one *Gossypium hirsutum* cultivar (ST 5288B2F) under two water regimes on July 2, and July 9, 2012 in Lubbock, TX (B). All values are means ± standard errors (n=5), and dates and water regime not sharing a common letter are significantly different ($P \leq 0.05$) according to Tukey's test.

Table 1. Effect of cultivar, water regime and days after stress, and the interaction between the factors on quantum yield of photosystem II (Φ_{PSII}), electron transport rate (ETR), chlorophyll *a*, chlorophyll *b*, and carotenoids in Marianna, AR.

Source of variation	Φ_{PSII}	ETR	Chl *a*	Chl *b*	Carotenoids
		µmol electrons $m^{-2}s^{-1}$	————	µg cm^{-2}	————
Cultivar					
DP 0912 B2RF	0.401 a †	236.56	8.43 b	2.11 b	2.63 b
PHY 499 WRF	0.360 b	215.76	8.96 a	2.35 a	2.67 ab
ST 5288B2F	0.381 ab	230.25	8.97 a	2.38 a	2.74 a
Water Regime					
Control	0.380	223.48	9.36 a	2.37 a	2.85 a
Water Stress	0.381	231.56	8.22 b	2.18 b	2.51 b
Days after Stress					
Seven	0.423 a	245.88 a	9.13 a	2.32 a	2.67
Fourteen	0.338 b	209.16 b	8.45 b	2.23 b	2.69
ANOVA					
Cultivar (C)	0.0434 *	ns	<0.0001 *	<0.0001 *	0.0113 *
Water Regime (WR)	ns	ns	0.0005 *	0.0066 *	0.0006 *
Days after Stress (DS)	<0.0001 *	0.0009 *	<0.0001 *	0.0007 *	ns
Interaction C x WR	ns	ns	ns	ns	ns
Interaction C x DS	ns	ns	ns	ns	0.0263 *
Interaction WR x DS	0.0414 *	ns	0.0409 *	ns	ns
Interaction C x WR x DS	ns	ns	ns	ns	ns

All values are means (n=5).
*Asterisks indicate significant difference in the treatments at P≤0.05.
† Letters close to values in column, within each factor (Cultivar, Water Regime, or Days of Stress), are indicated to compare significant difference at P≤0.05.

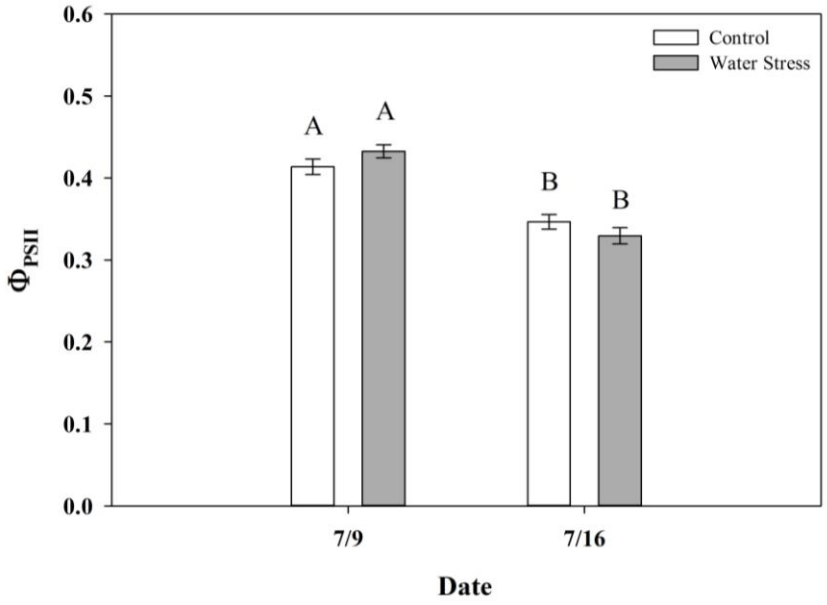

Figure 2. Interaction of water regime (well-watered control and water-deficit stress) and days after stress (July 9 and July 16) on quantum yield of photosystem II (Φ_{PSII}) of cotton plants in Marianna, AR in 2013. All values are means ± standard error (n=5). Bars not sharing a common letter are significantly different ($P \leq 0.05$) according to Tukey's test.

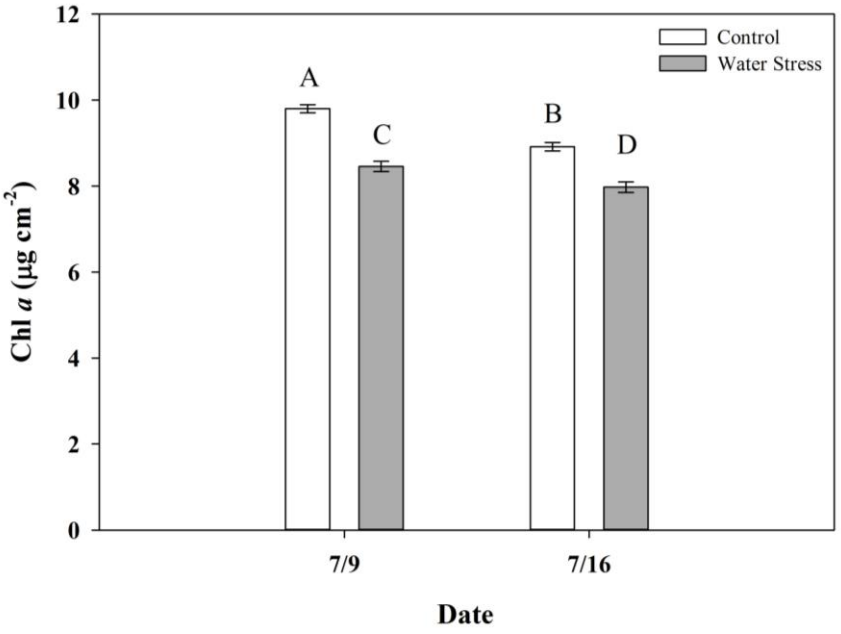

Figure 3. Interaction of water regime (well-watered control and water-deficit stress) and days after stress (July 9 and July 16) on concentration of chlorophyll *a* (µg cm^{-2}) of cotton plants in Marianna, AR in 2013. All values are means ± standard error (n=5). Bars not sharing a common letter are significantly different ($P \leq 0.05$) according to Tukey's test.

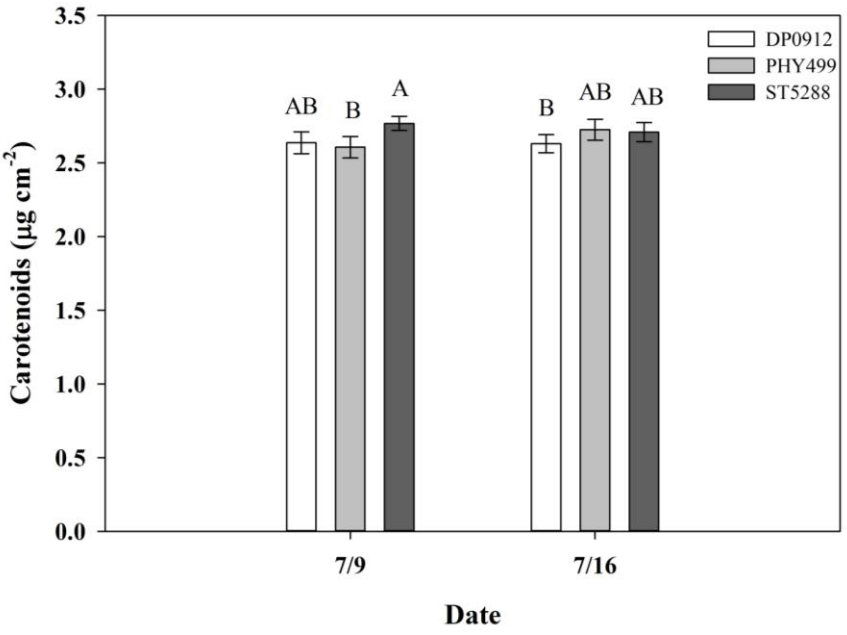

Figure 4. Interaction of cultivar (DP 0912 B2RF, PHY 499 WRF, and ST 5288B2F) and days after stress (July 9 and July 16) on concentration of carotenoids (µg cm^{-2}) of cotton plants in Marianna, AR in 2013. All values are means ± standard error (n=5). Bars not sharing a common letter are significantly different ($P \leq 0.05$) according to Tukey's test.

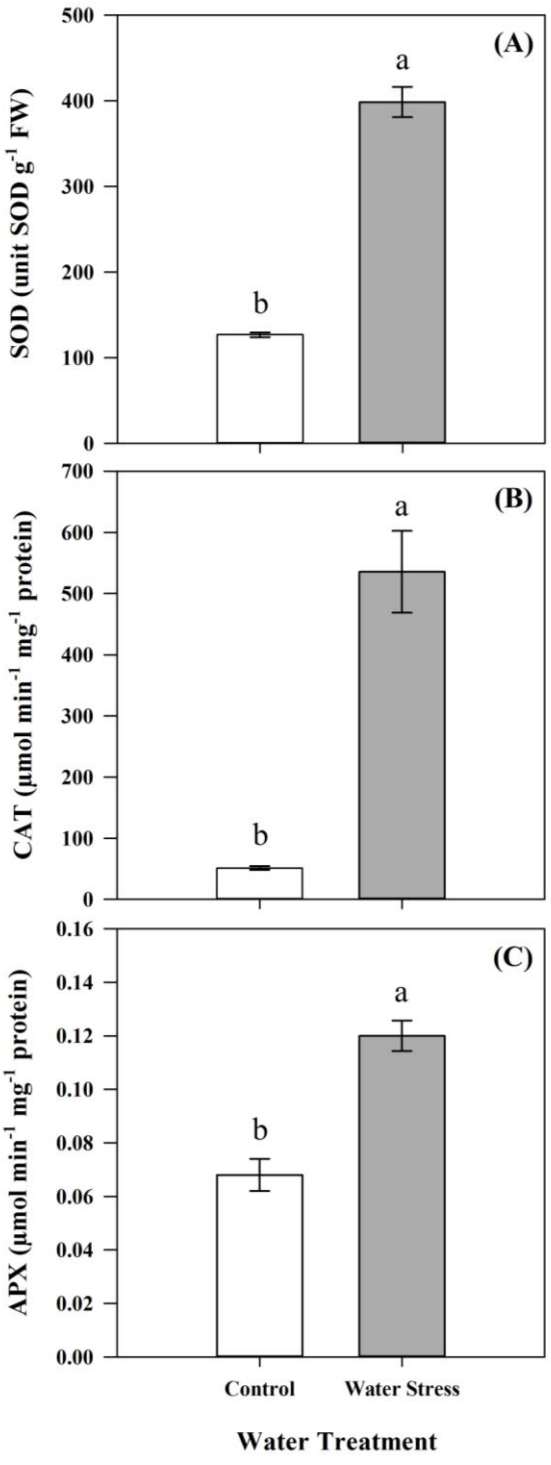

Figure 5. Effect of water regime (well-watered control and water-deficit stress) on activities of the enzymes superoxide dismutase (SOD, unit SOD g^{-1} FW) (A), catalase (CAT, µmol min^{-1} mg^{-1} protein) (B), and ascorbate peroxidase (APX, µmol min^{-1} mg^{-1} protein) (C) in the *Gossypium hirsutum* cultivar (ST 5288B2F) on July 9, 2012 in Lubbock, TX. All values are means ± standard error (n=5). Bars not sharing a common letter are significantly different ($P \leq 0.05$) according to Tukey's test.

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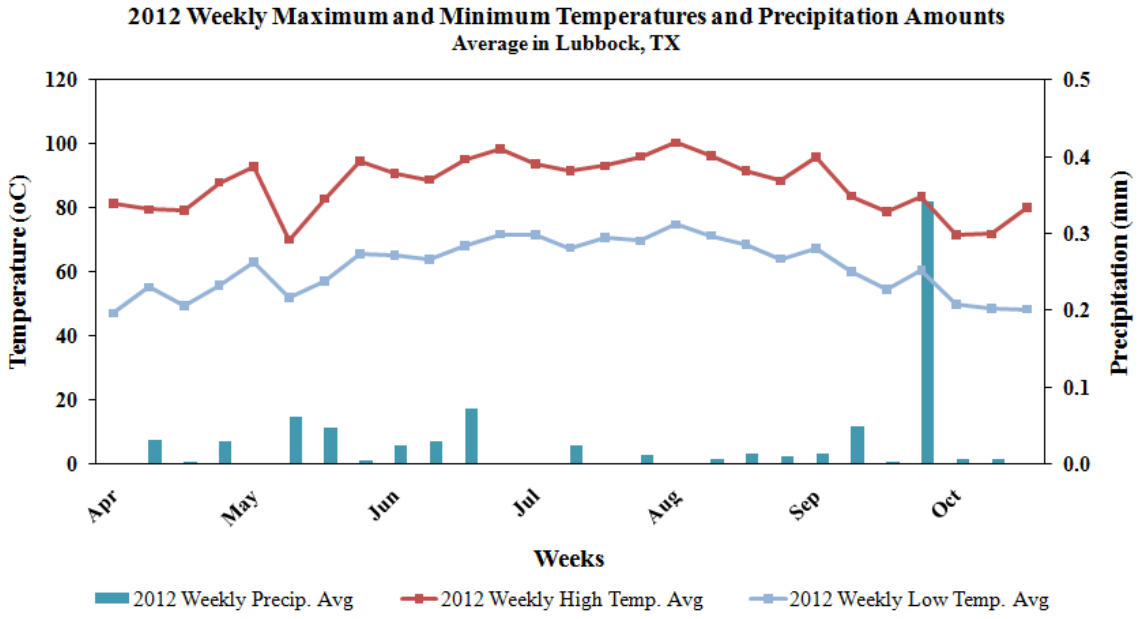
APPENDIX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Average in Lubbock, TX
Temperature (oC)
Precipitation (mm)
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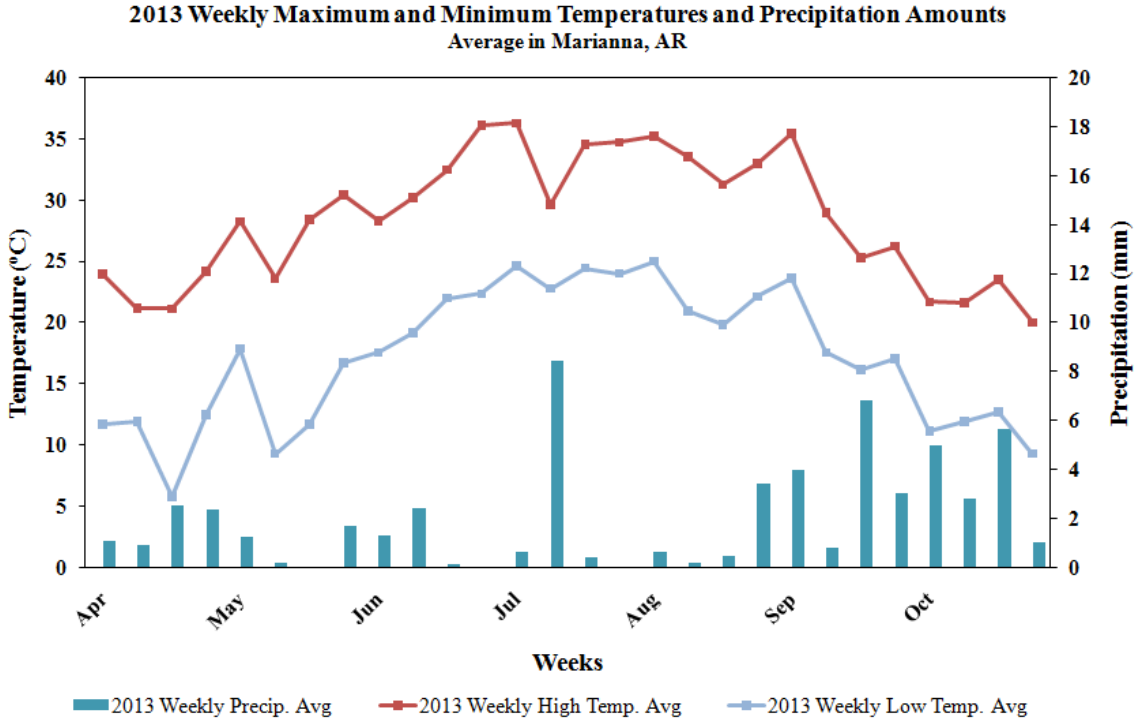
100
80
60
40
20
0
0.5
0.4
0.3
0.2
0.1
0.0
Apr
May
Jun
Jul
Aug
Sep
Oct
Weeks
2012 Weekly Precip. Avg
2012 Weekly High Temp. Avg
2012 Weekly Low Temp. Avg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Average in Marianna, AR
Temperature (°C)
Precipitation (mm)
40
35
30
25
20
15
10
5
0
20
18
16
14
12
10
8
6
4
2
0
Apr
May
Jun
Jul
Aug
Sep
Oct
Weeks
2013 Weekly Precip. Avg
2013 Weekly High Temp. Avg
2013 Weekly Low Temp. Avg

VI. ASSESSING PHOTOSYSTEM II QUANTUM YIELD HEAT TOLERANCE AS A FACTOR CONTRIBUTING TO DROUGHT TOLERANCE IN CONTRASTING COTTON GENOTYPES

ABSTRACT

Differences in tolerance to drought and heat stresses exist among cotton species and between modern and obsolete genotypes. However, it is not clear if increases in thermostability under water deficit are associated with genotypic differences in drought tolerance. Therefore, the objective was to identify differences in heat tolerance and physiological acclimation of contrasting cotton genotypes under water-deficit stress and recovery conditions. The experiments were conducted in growth chambers with three contrasting cotton genotypes and well-watered and water-stressed regimes. Heat tolerance was accessed through maximum quantum yield of Photosystem II at temperatures 25°C to 45°C and measurements of stomatal conductance and electrolyte leakage. Differences in heat tolerance and physiological acclimation exist among the genotypes under water-deficit stress and recovery. Pima 32 showed higher heat tolerance, improved stomatal conductance and lower electrolyte leakage after recovery. Siokra L23 was relatively heat sensitive and showed moderate recover in stomatal conductance and electrolyte leakage after plants were re-watered. DP 0912 was the least heat tolerant; however, it exhibited lower electrolyte leakage at recovery. In conclusion, thermostablity under water-deficit stress was associated with drought tolerance of genotypes, with Pima 32 having the highest heat tolerance acclimation in response to water-deficit stress, followed by Siokra L23 and DP 0912.

INTRODUCTION

Water-deficit stress is the most restricting factor to plant development and yield on a global scale due to limiting water supplies and climate variability. The severity and duration of drought as well as plant growth stage and genotype determine the effects of water stress in plants (Kramer and Boyer, 1995). Plants are usually exposed to drought-recovery cycles throughout the season and the effects of these episodic droughts on crop productivity depend on the plant's ability to prevent cellular damage during the stress and to recover after relief of the stress (Munne-Bosch and Penuelas, 2003; Blum and Ebercon, 1981).

Drought and heat stresses commonly co-occur in the field which makes control of plant water use more difficult (Saranga et al., 2001). Stomatal closure is one of the primary plant responses to water limiting conditions. It limits evaporative cooling of leaves, increases leaf temperature and reduces heat avoidance (Lu et al., 1994). Cotton plants have developed mechanisms to ensure their survival under water limiting conditions, such as stomatal closure and osmotic adjustment (Loka et al., 2011). Although differences in drought tolerance have been shown to exist between modern and obsolete genotypes (Nepomuceno et al., 1998; Oosterhuis and Wullschleger, 1987), the heat tolerance acclimation of PSII as a characteristic of drought tolerant cotton genotypes has not been explored.

Photosynthesis in leaves is reduced under drought stress due to stomatal closure which results in lower CO_2 internal concentrations, and decreased CO_2 fixation (Cornic, 2000). Photosystem II (PSII) is the initial complex in the photosynthetic electron transport chain, and is responsible for oxidation of water and generation of molecular oxygen (Lambers et al., 2008). Its efficiency and stability can be measured by means of maximum quantum yield of PSII (F_v/F_m). Thus, F_v/F_m has served as an indicator of injuries in PSII complex caused by stress conditions

(Rees et al., 1990; Krause and Weis, 1991; Lazar and Naus, 1998). Previous research has established that F_v/F_m is tolerant to both drought and high temperature conditions (Pettigrew, 2004; Lawlor and Tezara, 2009; Snider et al., 2013). Snider et al. (2013) recently reported that PSII heat tolerance was greatest for rain fed *G. hirsutum* plants at times during the growing season coinciding with maximal drought stress exposure. This suggests that increased PSII heat tolerance may be part of the acclimation response to water deficit, although controlled environment studies demonstrating improved PSII heat tolerance in drought stressed, relative to control plants, are limited in *G. hirsutum*. *Gossypium hirsutum* cv. Siokra L23 is regarded as one of the most drought tolerant Upland cotton cultivars available, as evidenced by gas exchange and yield responses to drought (Stiller et al., 2005). However, the possibility that heat tolerance acclimation could partially account for improved performance in Siokra L23 under drought is unexplored. Finally, *G. barbadense* (Pima cotton) is grown under extremely high temperature conditions in the southeastern United States, and when compared side-by-side in the field with *G. hirsutum*, *G. barbadense* leaves have much lower stomatal conductance and higher foliage temperatures (Lu et al., 1997). However, to our knowledge, studies evaluating differences in PSII heat tolerance between Pima and Upland cotton genotypes in response to water deficit are also non-existent.

Considerable variation in tolerance to drought and heat stresses exists between cotton species, *Gossypium hirsutum* and *G. barbadense*, and between modern and obsolete genotypes (Saranga et al., 1998; Bibi et al., 2008; Brown and Oosterhuis, 2010). The development of new drought-tolerant cultivars has been hampered by lack of knowledge on physiological mechanisms of utilized by plants to cope under water limiting conditions (Saranga et al., 2001). Progress has been made in developing high-yielding cotton cultivars; however, this selection has

narrowed the genetic variability for drought tolerance (Campbell et al., 2012). Some obsolete genotypes have traits for drought tolerance and they could be used in cotton breeding programs to assist in selection of modern cultivars widely adapted. We hypothesize that different physiological responses will exist among contrasting cotton genotypes exposed to water-deficit stress and that more drought tolerant genotypes will exhibit the greatest PSII thermostability. Therefore, the objective of this study was to identify differences in heat tolerance and physiological acclimation of contrasting cotton genotypes under water-deficit stress and recovery conditions.

MATERIALS AND METHODS

Location, plant material, and sampling protocol

An experiment was conducted in 2012 and repeated in 2013 at the Altheimer Laboratory, University of Arkansas in Fayetteville, Arkansas. The experimental design was a completely randomized design with five replications. Treatments consisted of three cotton genotypes, DP 0912 B2RF, Pima 32, and Siokra L23 (Table 1) and two water regimes, a well-watered control and a water-stressed treatment. Each pot with a single plant represented one experimental unit. Pima 32 is a *Gossypium barbadense* that has no introgressed genes from Upland cotton (Cornish et al., 1991).

Cotton genotypes were planted in 2-L pots filled with Sunshine potting media (Sun Gro Horticulture Distribution Inc., Bellevue, WA) in a large growth chamber (Model PW36, Conviron, Winnipeg, Canada). Temperatures of 32/24°C (day/night), 14h photoperiod, 60% relative humidity, and a typical diurnal pattern of the photosynthetically active radiation (PAR) with the highest PAR (850 µmol m^{-2} s^{-1}) between 10:00am and 2:00pm were maintained in the

growth chamber. The plants were watered once daily with half-strength Hoagland's nutrient solution (Hoagland and Arnon, 1933) to maintain adequate moisture until the appearance of floral buds (pinhead square stage) approximately 35 days after planting. At this stage, water stress was imposed by withholding water from the water-stressed plants until stomatal conductance (g_s) reached approximately 20 mmol $m^{-2}s^{-1}$ indicating that the stomatal mechanism was virtually ceased. Well-watered control plants received an optimum quantity of water throughout the duration of the experiment and the typical pattern of g_s in well-watered and drought stressed plants is presented in Figure 1. Once the water-stressed plants reached the required stress, samples for electrolyte leakage and maximum quantum yield of photosystem II (F_v/F_m) measurements were collected using the fourth uppermost fully expanded main-stem leaf of all 30 plants. After the measurements and samples were taken, the stressed plants were re-watered. Twenty-four hours after re-watering, stomatal conductance, electrolyte leakage and maximum quantum yield of photosystem II (F_v/F_m) were measured on the third uppermost fully expanded main-stem leaf for the recovery evaluation.

Stomatal conductance

Stomatal conductance was measured daily starting at the first day of the stress until the recovery using a steady-state leaf porometer (SC-1 Leaf Porometer, Decagon, Pullman, Washington, USA) on the abaxial surface of fourth uppermost fully expanded main-stem leaves. Measurements were taken between 12:00pm to 2:00pm with two readings per leaf. The results were expressed as mmol H_2O $m^{-2}s^{-1}$.

Electrolyte leakage

Electrolyte leakage was measured according to methodology described by Martineau et al. (1979) with modifications. Five leaf discs were punched from similar interveinal area of the fourth uppermost fully expanded main-stem leaf and placed in closed vials filled with 10 mL deionized water and incubated at 25°C for 48 h in the dark. After this period, the electrical conductivity of the solution (L_1) of all samples was determined using a Single Probe Conductivity Meter (Thermo Orion Model 115, Beverly, Massachusetts, USA). Subsequently, the vials were placed in a water bath at 100°C for ten minutes to obtain the maximum leakage of the leaf discs. Electrical conductivity was measured again (L_2) after the samples equilibrated to 25°C. The electrolyte leakage was calculated as: (L_1/L_2) * 100. Results were expressed in percentage.

Maximum quantum yield of photosystem II (F_v/F_m)

Leaves were collected from the fourth main-stem node below the plant terminal, wrapped in a moist paper towel and stored in ziploc plastic bags in the dark for 24h in order to dark adapt. Then, 5 cm^2 leaf segments were cut from each leaf sample and placed on a moist filter paper in contact with a thermoelectric heating/cooling block (Snider et al., 2010). The temperature of the thermal block was adjusted to 25°C and leaf segments were allowed to incubate for five min. After the incubation period, maximum quantum yield of photosystem II (F_v/F_m) was measured using the OS5p Chlorophyll Fluorometer (Opti-Sciences, Hudson, New Hampshire, USA). Briefly, F_0 was measured under a low-intensity modulation light source immediately prior to exposure of the leaf sample to a saturating light pulse for 0.8 s to determine F_m. F_v/F_m was calculated as follows: $F_v/F_m=[(F_0-F_m)/F_m]$. This procedure was repeated for the temperatures 30,

35, and 40°C in 2012 and 30, 35, 40 and 45°C in 2013. Thermotolerance was assessed by quantifying percent decline in F_v/F_m for individual leaves at 40°C (2012 and 2013) and 45°C (2013) in relation to 25°C (Figure 2). Higher % decline is indicative of greater sensitivity to high temperature. To assess the impact of drought stress alone on PSII function in each cotton genotype, the F_v/F_m value obtained at 25°C (prior to increasing leaf temperature) was recorded for each leaf.

Statistical analysis

As similar trends and no significant differences were observed for stomatal conductance, electrolyte leakage, and F_v/F_m at 25°C in the two years of experiment, the results were pooled and the means were taken. A two-way analysis of variance (ANOVA) was performed for each sample date using JMP Pro 11 (SAS Institute, Cary, NC). The treatments genotype and water regime were considered as fixed effect. The blocks and the block x treatment interaction were considered as random effect. Treatment means were separated using Tukey's HSD test at the 0.05 probability level.

RESULTS

Stomatal conductance

Stomatal conductance was measured daily from the first day of the stress until one day after the water-stressed plants were re-watered, as shown in Figure 1. Water was withheld in the water-stressed plants until g_s reached approximately 20 mmol $m^{-2}s^{-1}$. Then, the plants were re-watered and the recovery was recorded one day after the water-stressed plants received the same

amount of water as the control plants. The stress period of all genotypes was four days in average.

Stomatal conductance was affected by genotype x water regime interaction (Table 2) at the last day of the stress and one day after recovery. Stomatal conductance of all genotypes was significantly decreased by the water-deficit stress (Figure 3a) when evaluated on the last day of the stress. Under well-watered conditions, Siokra L23 showed the highest g_s followed by DP 0912 and Pima 32. However, there was no significant difference in g_s of the water-stressed plants among the genotypes, and the overall average was approximately 17 mmol m^{-2} s^{-1}. Stressed plants of all genotypes increased stomatal conductance at recovery compared with the last day of the stress (Figure 3). However, the increase was not sufficient to reach stomatal conductance rates of the well-watered plants, except for Pima 32 which had the stomatal conductance of the stressed plants statistically similar to the well-watered plants (Figure 3b). Siokra L23 showed the highest g_s compared with the other genotypes under both well-watered and water-stressed conditions at recovery.

Electrolyte leakage

At the last day of the stress, electrolyte leakage was affected by genotype and water regime treatments (Table 2). Electrolyte leakage was significantly higher in the leaves of water-stressed plants of Pima 32 compared with the control, while no significant difference was found between water regimes in Siokra L23 and DP 0912 (Figure 4a). Under well-watered conditions, Siokra L23 showed higher electrolyte leakage than DP 0912. However, there was no statistically difference in electrolyte leakage among the genotypes under water-stressed conditions. At recovery, electrolyte leakage was affected only by genotype (Table 2). Electrolyte leakage was

statistically similar in both water regimes for all genotypes after a short period of recovery (Figure 4b). Electrolyte leakage was higher for Siokra L23 than Pima 32 and DP 0912 under well-watered conditions. However, under water-stress, there was no significant difference in the electrolyte leakage among the genotypes studied. A short period of recovery assisted water-stressed plants from Siokra L23 and Pima 32 to reduce electrolyte leakage 13% and 18%, respectively (Figure 4). DP 0912 was not influenced by re-watering the stressed plants, maintaining similar % electrolyte leakage of the cells in the control and water-stressed plants for both sample dates.

Maximum quantum yield of photosystem II (F_v/F_m)

Maximum quantum yield of photosystem II (F_v/F_m) at 25°C was affected by genotype at the last day of the stress and at recovery and by water regime at the last day of the stress (Table 2). DP 0912 showed the highest F_v/F_m under both water regimes compared with the other genotypes at the last day of the stress (Figure 5a). DP 0912 and Pima 32 had the F_v/F_m significantly decreased by the water-deficit stress, while the F_v/F_m of Siokra L23 was not significantly affected by the water-deficit stress. At recovery, DP 0912 was the only genotype that had significantly lower F_v/F_m in the stressed plants compared with the well-watered plants (Figure 5b).

When heat tolerance was assessed at 40°C, genotype and water regime treatments influenced thermotolerance differently depending upon year and sample date (Table 3). Heat tolerance was either unaffected by water-stressed treatment in Siokra L23 (Figure 6a-c) or decreased (decline indicates more heat sensitive) following a short recovery period (Figure 6d) only in 2013. However, there were a few consistent trends across sample dates and years. DP

0912 was always more (numerically or statistically) heat sensitive at 40°C in water-stressed plants whether plants were sampled during water-deficit exposure or following a short recovery period. Percent decline in F_v/F_m for Pima 32 was unaffected by water regime treatment, regardless of sample date, and Pima 32 was, in all instances, the most heat tolerant or statistically equivalent to the most heat tolerant cultivar at any sample time (Figure 6).

When leaf temperatures were increased to 45°C in 2013, a significant interaction was observed between genotype and water regime treatment for % decline in F_v/F_m (Figure 7). Under well-watered conditions, DP 0912 exhibited the greatest heat sensitivity, as evidenced by having the highest % decline in F_v/F_m at 45°C (Figure 7a), whereas exposure to water deficit slightly enhanced thermotolerance for DP 0912 in both sample dates. The reverse trend was observed for Siokra L23, where heat tolerance was higher under well-watered conditions than water-stressed conditions. Siokra L23 exposed to either water regime was more heat tolerant than DP 0912. Pima 32 exhibited the greatest heat tolerance of any genotype evaluated (except for Siokra L23 under well-watered conditions which was equally heat tolerant), and thermotolerance was unaffected by irrigation treatment for this genotype.

Similar trends were observed following recovery (Figure 7b), where DP 0912 was the least heat tolerant cultivar, and heat tolerance was higher in previously drought-stressed leaves of DP 0912 than those maintained under well-watered conditions. In contrast, heat tolerance was lower in Siokra L23 leaves that had been previously exposed to drought stress before a brief recovery period than Siokra L23 leaves that had been maintained under well-watered conditions throughout the experiment. Siokra L23 under well-watered conditions and Pima 32, under either irrigation treatment, were the most heat tolerant following the recovery period.

DISCUSSION

Stomatal function is known to be one of the first mechanisms affected by the onset of water-deficit stress (Hsiao, 1973). Our results indicate that stomatal conductance of all genotypes studied was lower under water-deficit stress compared with the well-watered control regardless of sample date, except for Pima 32 which showed stomatal conductance of water-stressed plants statistically equivalent to well-watered plants at recovery. Several reports on cotton plants cultivated in pots under drought stress demonstrated that stomatal closure increases considerably under moderate or severe water-deficit stress (Carmo-Silva et al., 2012; Li et al., 2012; Wullschleger and Oosterhuis, 1990; Boyer, 1970). Stomatal closure prevents water loss through stomata. Siokra L23 had the highest stomatal conductance among the genotypes studied under well-watered control conditions. This might be due to its Australian origin, which confers tolerance to low water availability. Also, Siokra L23 has okra leaf, which has smaller boundary layer and higher evaporative cooling capacity (Heitholt and Meredith, 1998). All the genotypes studied increased stomatal conductance of the stressed plants after re-watering, suggesting that the plants had started recovering from the water stress period. Stomatal conductance of Pima 32 water-stressed plants had similar rates to the control, indicating that this genotype was able to adjust the stomatal mechanism and recover from a short period of water scarcity.

Cellular membrane thermostability has been used to assess heat tolerance in plants (Martineau et al., 1979; Bibi et al., 2008). Membrane permeability increases as the injury in the leaf tissue occurs in response to exposure to high temperatures and as a result, electrolytes diffuse out of the cells. The amount of electrolyte leakage can be determined by electrical conductance evaluation. This measurement has also been used to evaluate the percentage of injury in cellular membrane of plants exposed to water-deficit stress (Lv et al., 2007; Lauriano et

al., 2000). Our results demonstrate that Pima 32 and DP 0912 had the lowest electrolyte leakage in all instances, except for Pima 32 under water-stress on the last day of the stress. This suggests that these two genotypes were able to adjust the damage caused by the water-deficit stress by recovering after the plants were re-watered. Electrolyte leakage of all genotypes was higher (numerically or statistically) under water-deficit stress on the last day of the stress. However, when the plants were re-watered, electrolyte leakage of all genotypes decreased to similar (or lower) rates to the control plants, indicating some recovery.

Heat tolerance can also be assessed in cotton genotypes by means of F_v/F_m, which is an indicator of heat sensitivity of PSII complex *in vivo* in plants (Krause and Weis, 1991). Data of % decline in F_v/F_m support the hypothesis that obsolete and modern genotypes differ in heat tolerance under water-stress conditions and after a short period of recovery. Pima 32 was consistently and statistically the most heat tolerant cultivar as the lower % decline of F_v/F_m indicates greater tolerance to high temperatures, and heat tolerance was unaffected by water regime. These findings indicate that Pima is innately heat tolerant, and may not need to acclimate to drought by increasing its heat tolerance. This is likely due to its origin from hot growing environment and already lower stomatal conductance and higher leaf temperatures than *G. hirsutum* as reported previously (Lu et al., 1997). Heat tolerance of Siokra L23 was either not influenced by water regime or more heat sensitive under water-stress conditions than well-watered control. Additionally, water-stressed Siokra L23 leaves increased heat sensitivity after a short period of recovery, suggesting that increasing heat tolerance in response to drought may not be a part of this genotype's mechanism for coping with drought since it was less heat tolerant when exposed to water-deficit stress conditions. DP 0912 was generally the least heat tolerant genotype under well-watered conditions, but consistently increased heat

tolerance following exposure to drought, similar to a previous report with this same cultivar (Snider et al., 2013). This indicates that increasing heat tolerance (acclimation) is an important mechanism that this particular cultivar uses to cope with water-deficit stress.

In conclusion, differences in heat tolerance and physiological acclimation exist among the genotypes studied under water-deficit stress and recovery conditions. Pima 32 was the most heat tolerant genotype and had higher recovery capacity in stomatal conductance and electrolyte leakage after the plants were re-watered. Siokra L23 was relatively heat sensitive and had stomatal conductance and electrolyte leakage reduced by water-deficit stress. DP 0912 was the least heat tolerant genotype under well-watered conditions; however, it increased heat tolerance in the water-stressed plants at recovery. Stomatal conductance in DP 0912 was greatly decreased by water-deficit stress. Overall, it appeared that the most obsolete genotype Pima 32 had superior heat tolerance acclimation, which was indicated as a characteristic of drought tolerant cotton genotypes, while Siokra L23 and the modern genotype DP 0912 were considered heat sensitive under both water regimes.

TABLES AND FIGURES

Table 1. Background information for the obsolete and modern cotton genotypes used in the experiments.

Genotype	Species	Origin	Leaf Type	Characteristic	Year of development
DP 0912 B2RF	*G. hirsutum*	Mid-South United States	Normal	Relative drought sensitive	2009
Pima 32	*G. barbadense*	California, West United States	Normal	Drought tolerant	1949
Siokra L23	*G. hirsutum*	Victoria, South-East Australia	Okra	Drought tolerant	1991

Table 2. Analysis of variance (ANOVA) of stomatal conductance, electrolyte leakage, and F_v/F_m at 25°C at the last day of the stress and one day of recovery.

Source of variation	d.f.	*F*-value					
		Stomatal conductance		Electrolyte leakage		F_v/F_m at 25°C	
		Last Day	Recovery	Last Day	Recovery	Last Day	Recovery
Genotype (G)	2	8.63 **	13.02 **	3.65 *	3.79 *	10.75 **	19.22 **
Water regime (WR)	1	71.27 **	29.35 **	6.38 *	0.97 ^{ns}	6.56 *	0.73 ^{ns}
G x WR		8.55 **	4.51 *	0.53 ^{ns}	1.29 ^{ns}	0.19 ^{ns}	0.72 ^{ns}

^{ns} nonsignificant; *Significant at $P < 0.05$; **Significant at $P < 0.001$.

Table 3. Analysis of variance (ANOVA) of percent decline in F_v/F_m at 40°C and 45°C at the last day of the stress and one day of recovery.

Source of variation	d.f.	*F*-value					
		[a] % F_v/F_m at 40°C				[b] % F_v/F_m at 45°C	
		Last Day	Recovery	Last Day	Recovery	Last Day	Recovery
		2012				2013	
Genotype (G)	2	12.65 **	10.02 **	50.06 **	49.04 **	125.13 **	76.41 **
Water regime (WR)	1	9.38 **	2.42 [ns]	3.88 [ns]	14.31 **	0.10 [ns]	9.65 **
G x WR		0.97 [ns]	0.60 [ns]	1.88 [ns]	36.59 **	9.69 **	25.16 **

[a] Percent decline in F_v/F_m at 40°C.
[b] Percent decline in F_v/F_m at 45°C.
[ns] = nonsignificant; *Significant at P < 0.05; **Significant at P < 0.001.

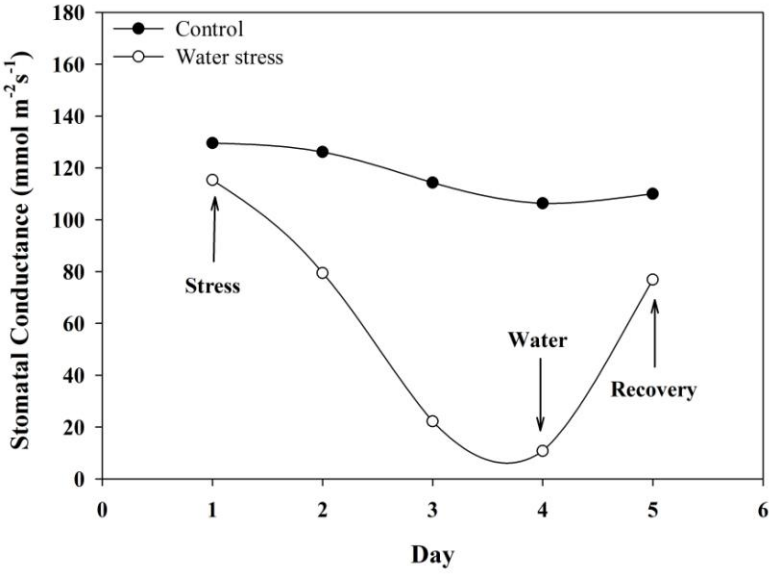

Figure 1. Stomatal conductance from the onset of the stress period until the first day of recovery of an individual leaf of *Gossypium barbadense* cv. Pima 32. Closed and open circles represent the stomatal conductance for each sample date in both water regimes, control and water stress. As indicated with arrows, water-deficit stress started when plants reached the pinhead square stage. Water was withheld from the water-deficit stress treatment until stomatal conductance reached 20 mmol $m^{-2}s^{-1}$. Then the stressed plants were re-watered and recovery measurements were taken one day after the water-stressed plants received the same amount of water as the control plants.

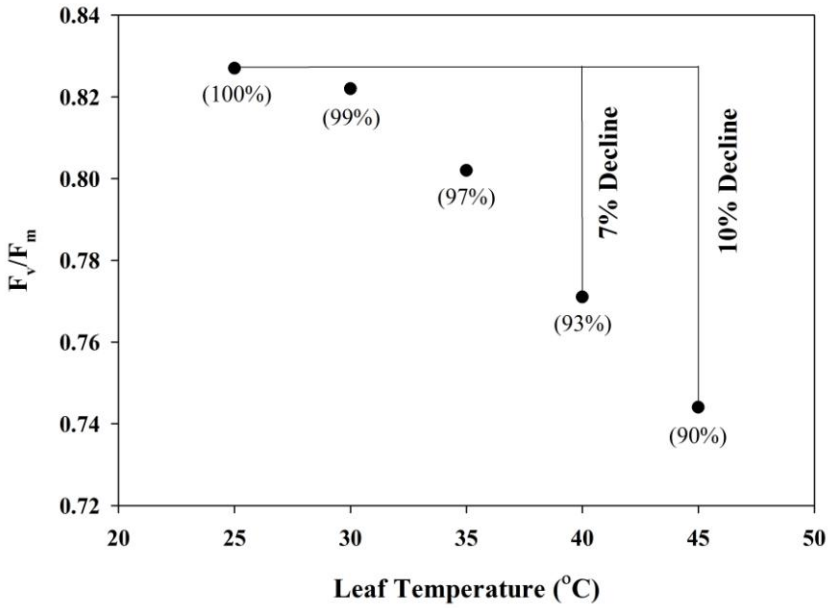

Figure 2. The response of F_v/F_m to leaf temperature for an individual leaf of growth chamber-grown *Gossypium hirsutum* cv. DP 0912 B2RF. The figure illustrates how % decline was calculated at 40 and 45°C in relation to 25°C.

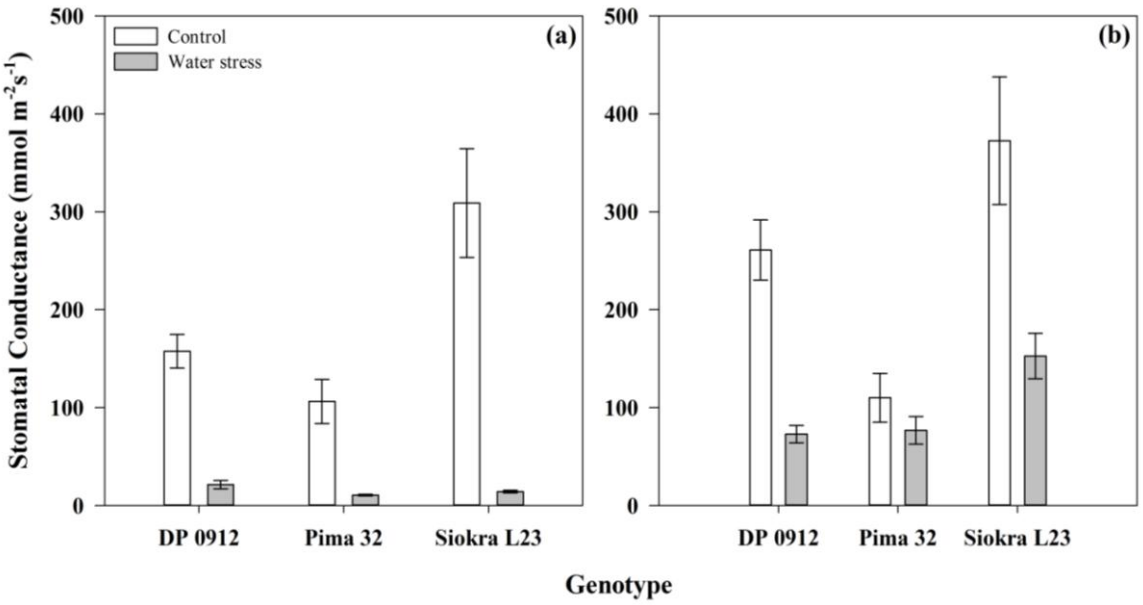

Figure 3. Stomatal conductance (mmol H_2O $m^{-2}s^{-1}$) on the last day of the stress (a) and first day of recovery (b) of three cotton genotypes (Siokra L23, DP 0912 B2RF, and Pima 32). All values are means ± standard error (n=5).

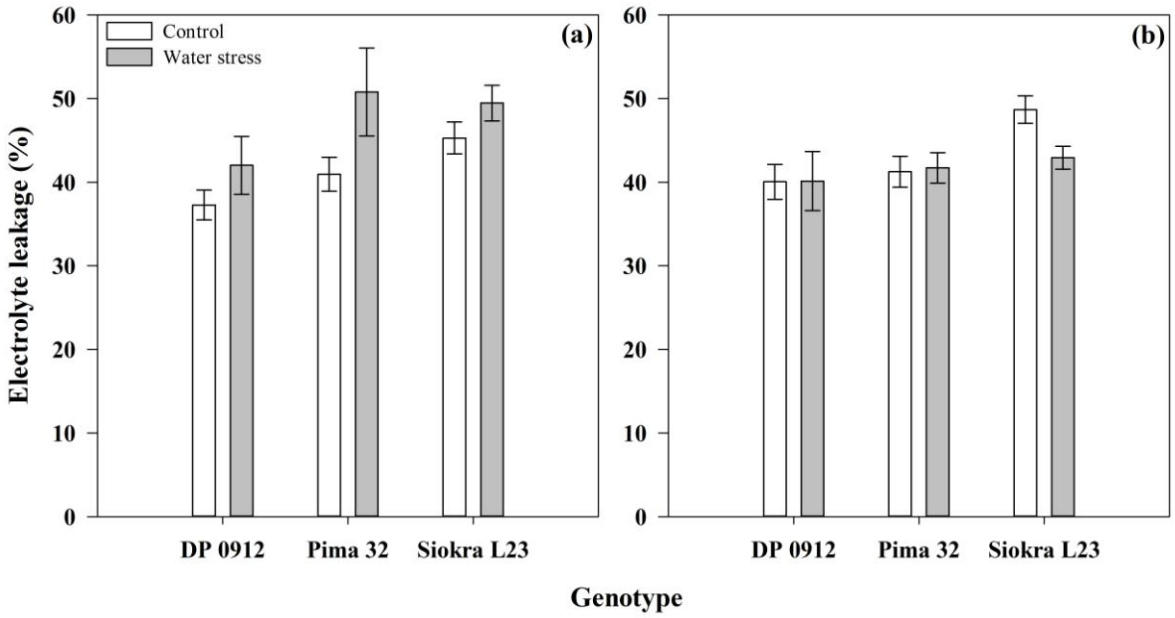

Figure 4. Electrolyte leakage (%) on the last day of the stress (a) and first day of recovery (b) of three cotton genotypes (Siokra L23, DP 0912 B2RF, and Pima 32). All values are means ± standard error (n=5).

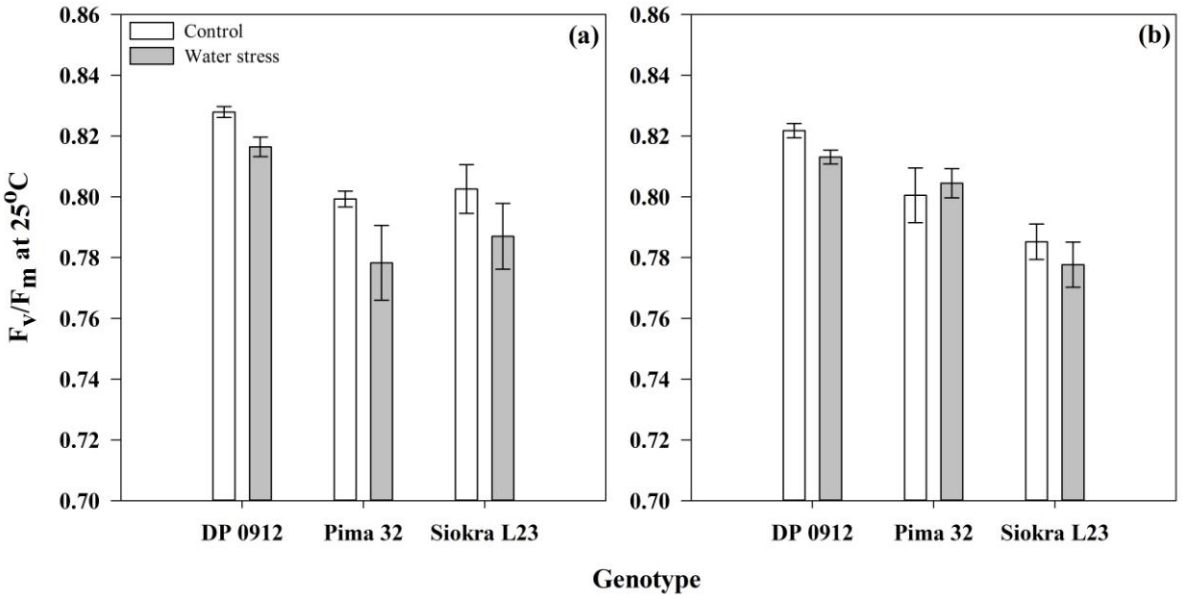

Figure 5. The response of F_v/F_m at 25°C to water-deficit stress on the last day of the stress (a) and first day of recovery (b) of three cotton genotypes (DP 0912 B2RF, Pima 32, and Siokra L23). All values are means ± standard error (n=5).

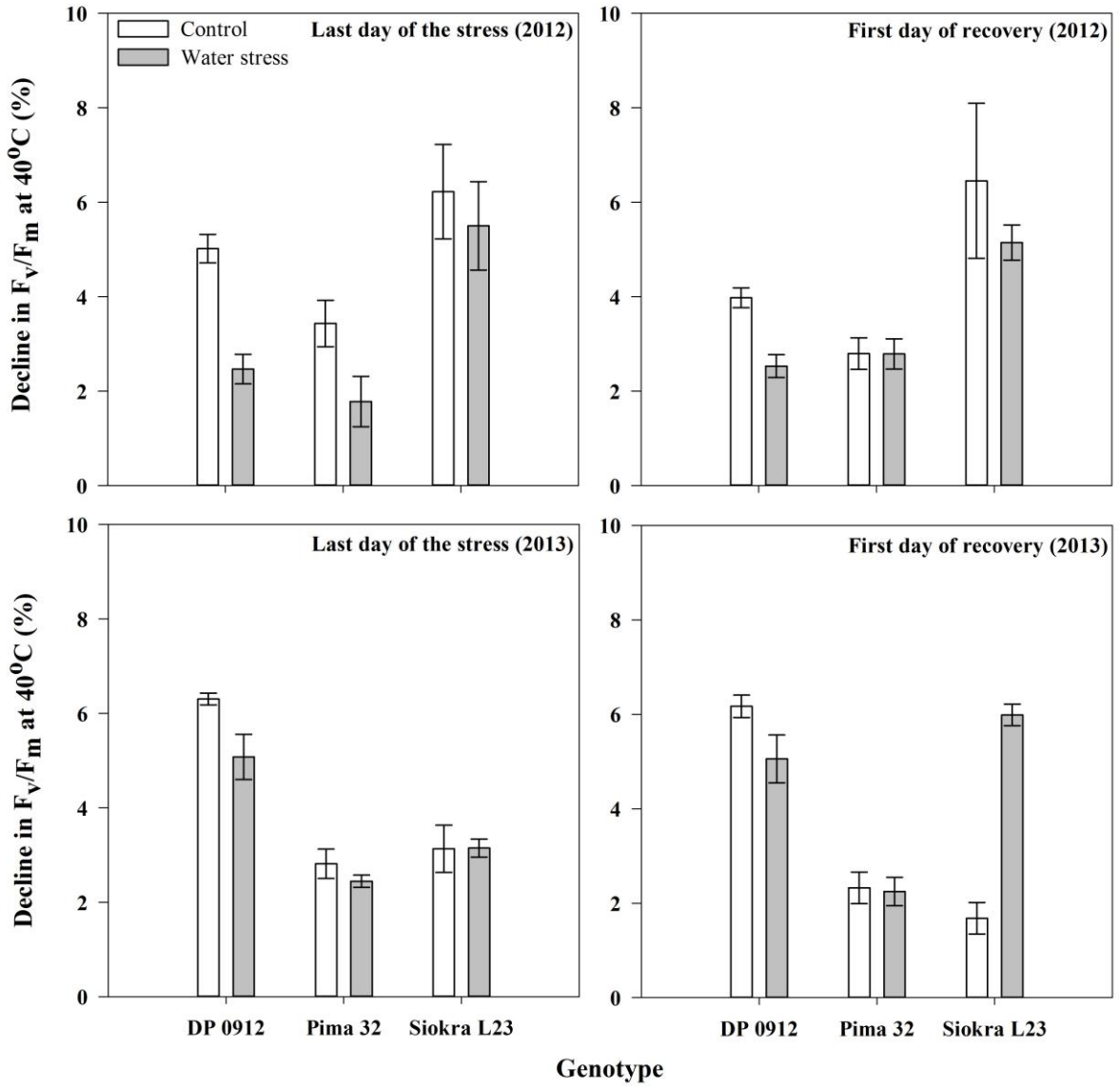

Figure 6. The effect of genotypes and water regime on the percent decline in maximum quantum yield of photosystem II (F_v/F_m) at 40°C on the last day of the stress and first day of recovery in 2012 and 2013. Decline in F_v/F_m represents the percent decline in F_v/F_m at 40°C in relation 25°C. All values are means ± standard error (n = 5).

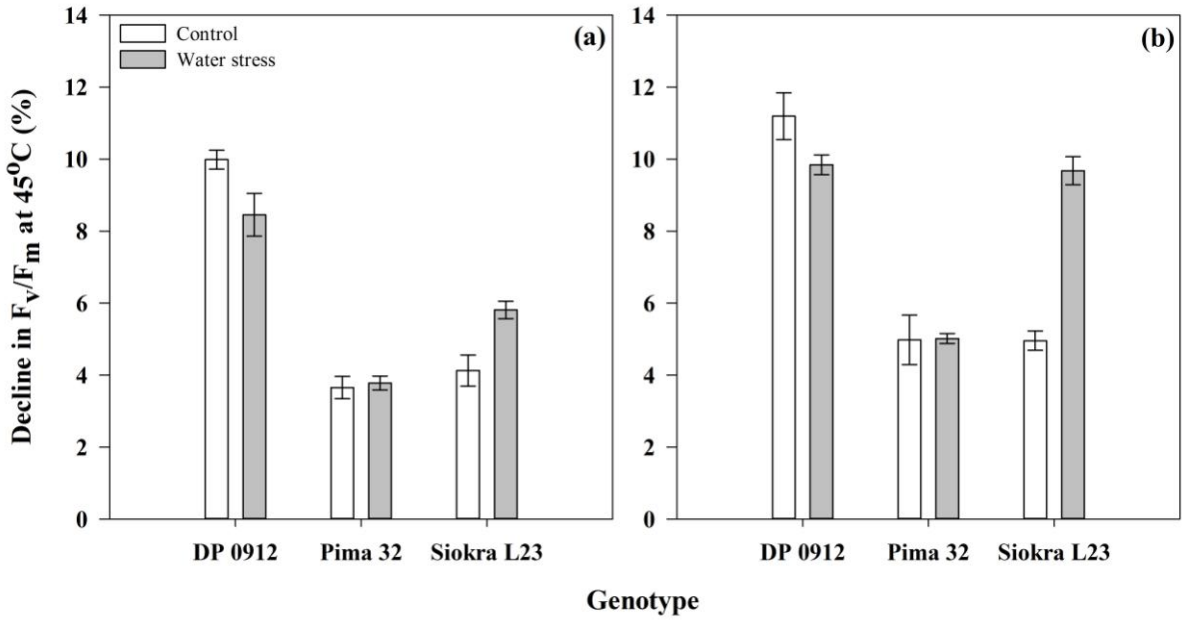

Figure 7. The effect of genotypes and water regime on the percent decline in maximum quantum yield of photosystem II (F_v/F_m) at 45°C at the last day of the stress (a), and first day of recovery (b) in 2013. Decline in F_v/F_m represents the percent decline in F_v/F_m at 45°C in relation to 25°C. All values are means ± standard error (n = 5).

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V. PHYSIOLOGICAL RESPONSE ON LEAVES OF MODERN COTTON CULTIVARS UNDER WATER-DEFICIT STRESS DURING THE FLOWERING STAGE

ABSTRACT

It has been well-established that water-deficit stress during flowering development impairs physiological processes, such as photosynthesis, in cotton (*Gossypium hirsutum* L.) plants leading to reduction in yield. Producers pursue drought tolerant cultivars in order to ensure yield production under water-deficit episodes throughout the season. Maintenance of leaf chlorophyll content and carbohydrate metabolism are indicators of drought tolerance in plants. However, information on chlorophyll content in leaves and carbohydrate metabolism in modern cotton cultivars is still limited. Therefore, the objectives of this study were to evaluate changes in carbohydrates concentrations in leaves and identify variation in drought tolerance among six modern cultivars subjected to water-deficit stress during the flowering development. Field experiments using six diverse modern cotton cultivars were conducted in New Deal, TX and Marianna, AR. Plants were subjected to water-deficit stress at the appearance of the white flower in the first sympodial fruiting position, and water was withheld for seven days in New Deal and fourteen days in Marianna. After seven days of stress (in New Deal and Marianna) and fourteen days of stress (in Marianna), soil water content was obtained from each plot, stomatal conductance and leaf chlorophyll content were measured in leaves from the fourth main-stem node below the plant terminal, and leaf samples were also collected for carbohydrates concentrations. Results indicated that the cotton cultivars did not differ in accumulation of carbohydrates within the same water regime, well-watered control, seven and fourteen days of water-deficit stress. The cultivars FM2484 and DP1044 showed relative drought tolerance under

mild water-deficit stress by improving green color of leaves and accumulating high concentrations of carbohydrates in the leaves of stressed plants, respectively.

INTRODUCTION

Cotton (*Gossypium hirsutum* L.) is one of the most important crops for fiber production in the U.S. and throughout the world. This crop has also a great contribution to food industries for livestock, dairy cattle and poultry (NCCA, 2015). Drought stress is one of the main factors affecting cotton plants limiting production worldwide (Osakabe et al., 2014). In general, cotton growers rely on rainfall throughout the season to supply the water requirement for production, as only approximately 35% of the U.S. cotton is grown under irrigation (Farahani and Munk, 2012), and in places such as Brazil and some African countries, irrigation is practically not used. Therefore, drought episodes reduce water availability for physiological and metabolic processes in the plants consequently affecting yield. Increased knowledge on drought tolerance of modern cotton cultivars is thus crucial for maintaining production in regions where water supply is limited.

Water-deficit stress alters several physiological processes in plants such as stomatal mechanism and synthesis of pigments as well as photosynthesis and transpiration (reviewed by Loka et al., 2011). The quantity of water used by cotton plants is related to the efficiency of physiological processes responsible for crop growth and yield (Deeba et al., 2012). Therefore, final cotton yield decreases due to lower photosynthetic efficiency in plants grown under drought stress.

Measurements of quantum efficiency of photosystem II and estimated chlorophyll content (SPAD index) are reported to be useful to identify differences of tolerance in plants

(Rong-hua et al., 2006). Other parameters such as changes in carbohydrates concentration in the plant can be also correlated to tolerance to drought stress (Kadkhodaie et al., 2013).

Carbohydrate metabolism of the plants is known to be altered under water-deficit stress resulting in accumulation of soluble sugar (Chaves, 1991). Expanded cotton leaves maintain exportation of fixed carbon even under low water availability in the cells, which is related to accumulation of soluble sugars leading to osmotic adjustment as an adaptation mechanism to tolerate stress conditions (Kadkhodaie et al., 2013; Chaves, 1991).

Modern cotton cultivars differ in tolerance to environmental stresses, including drought (Brown et al., 2004). Thus, the ability of a cultivar to maintain cell turgor for ongoing stomatal conductance and other physiological and metabolic processes such as photosynthesis and carbohydrates metabolism are indicative of potential to tolerate drought periods and maintain productivity under water-deficit stress (Mir et al., 2012). However, the reliability of these measurements as indication of drought tolerance varies among the genotypes as they differ in mechanisms to tolerate stress.

In the cotton crop, after seed germination and stand formation are concluded, the flowering stage is the most sensitive to water-deficit conditions reducing significantly final yield due to boll establishment during this stage (reviewed by Loka et al., 2011). The understanding of physiological and metabolic responses to water-deficit stress during flower and boll development is important for identification of cultivars with tolerance to drought periods. Therefore, the objectives of this study were to quantify changes in concentrations of carbohydrates in cotton leaves and identify differences in drought tolerance among six diverse cultivars grown under water-deficit stress during flowering. We hypothesized that low water availability during the flowering stage would induce accumulation of solutes through higher concentrations of

carbohydrates in the plants and cotton cultivars would differ in drought tolerance when plants experience a water-deficit stress during flowering.

MATERIALS AND METHODS

Field experiments were conducted at the New Deal Research Farm from Texas Tech University in New Deal, TX (N 33°44'13", W 101°43'58"), and at the Lon Mann Cotton Research Station in Marianna, AR (N 34°43'50", W 90°45'34") in 2014.

Treatments consisted of six cotton (*Gossypium hirsutum* L.) cultivars, DP 0912 B2RF (Delta and Pine Land, Monsanto Company, St. Louis, MO), DP 1044 B2RF (Delta and Pine Land, Monsanto Company, St. Louis, MO), PHY 499 WRF (PhytoGen, Dow AgroSciences, Indianapolis, IN), FM 2484 B2F (FiberMax, Bayer CropScience, Lubbock, TX), ST 4946 GLB2 (Stoneville, Bayer CropScience, Lubbock, TX), and NG 1511 B2RF (NexGen, Americot Inc., Lubbock, TX) and two water regimes, well-watered control and water-deficit stress imposed at appearance of white flowers in the first sympodial fruiting position (flowering stage), with four and five replications at New Deal and Marianna, respectively. Seeds were sown at a seeding rate of approximately 10 seeds m^{-1} in a Pullman clay loam (fine, mixed, superactive, thermic Torrertic Paleustoll) soil on June 2nd in New Deal and a Memphis silt loam (fine-silty, mixed, active, thermic Typic Hapludalfs) soil on May 07th in Marianna. A total of 60 plots in Marianna and 48 plots in New Deal, each composed of 4 rows with a row spacing of 0.96 m were used for the experiments. To further characterize site conditions, the average weekly maximum and minimum temperatures, and precipitation of each location throughout the season are presented in Appendix.

Fertilization was performed according to soil tests prior to planting and recommended rates for cotton. Herbicide and pesticide applications were also applied according to Texas A&M AgriLife extension recommendations (New Deal) and University of Arkansas Cooperative Extension Service recommendations (Marianna). Mepiquat chloride was applied as needed to control vegetative growth and all plots received identical applications.

The whole field was irrigated with a subsurface drip system in New Deal according to Texas Tech University New Deal farm's weather station and a furrow system in Marianna according to University of Arkansas Cooperative Extension Service recommendations until appearance of white flowers in the first sympodial fruiting position. When plants reached the flowering stage, August 14th in New Deal and July 21st in Marianna, water was withheld from the water-stress treatment for seven days in New Deal and fourteen days in Marianna.

Field measurements of stomatal conductance, leaf chlorophyll content, and leaf samples for laboratory determinations of carbohydrates concentrations were performed on the fully expanded main-stem leaf on the fourth node below the apical meristem from the two middle rows of each plot seven days (New Deal and Marianna) and fourteen days (Marianna) after irrigation was withheld.

Stomatal conductance

Stomatal conductance rates were measured between 1100 h to 1400 h in five leaves per plot and two readings per leaf (which were averaged) due to the small surface area of the cuvette (6.35 mm^2) (n=10) using a steady-state leaf porometer (SC-1 Leaf Porometer, Decagon, Pullman, WA) on abaxial surface of fully expanded main-stem leaf on the fourth node below the apical meristem. The results were expressed as mmol H_2O $m^{-2}s^{-1}$.

Leaf chlorophyll content

Leaf chlorophyll content (SPAD index) was measured *in-situ* on five fully expanded main-stem leaf on the fourth node below the apical meristem per plot and two readings per leaf (which were averaged) due to the small surface measurement area (2 mm x 3 mm) (n=10) using the SPAD-502 portable chlorophyll meter (Minolta Co. LTD., Osaka, Japan).

Carbohydrates concentration

Soluble carbohydrate content was measured according to Hendrix (1993) with modifications. Leaf samples were oven dried for 5 days at 50°C and then ground with a mortar and pestle. Forty mg of the ground tissue were extracted 3 times with 80% aqueous ethanol (800 mL ethanol / L) and the samples were centrifuged after each extraction at 5000 rpm and then the fractions were pooled. Active charcoal was added to the fractions to remove substances that could alter the carbohydrate measurements and the samples were centrifuged at 3500 rpm. The supernatant was immediately stored in -80°C for determination of sucrose and hexose (fructose and glucose) and the residue was stored in fridge for 20 h at 4°C for determination of starch.

For starch determination, 0.5 mL of potassium hydroxide (KOH) was added to each tube and they were placed in a water bath for 1 h. After the samples were cooled down, the pH was adjusted to 7 using 1M acetic acid. Then 50 µL of TRIS buffer and 100 µL a-amylase were added and samples were placed in water bath at 85°C for 30 min. After this, 0.5 mL amyloglucosidase was added and samples were incubated in water bath at 55°C for 60 min. Enzyme reaction was stopped by heating the samples in a thermoblock at 100°C for 5 min. Deionized water was added to the samples to reach final volume of 1.5 mL and samples were centrifuged in 10000 rpm for 10 min. For starch readings, 20 µL of the sample, 10 µL of water

and 100 µL of the Glucose Assay Reagent were pipetted in each well of a microtitration plate. Plate was incubated at 30°C for 15 min and absorbance was read at 340 nm.

For soluble sugars, the glucose (HK) assay kit from Sigma (Sigma Chemical Company, St. Louis, MO) was used. A 20 µL aliquot of each extract was pipetted into a well of a microtitration plate and the plate was incubated at 50°C for 40 min to evaporate ethanol. For glucose readings, 10 µL of water was added to each well along with 100 µL of glucose assay reagent and the plate was incubated again for 15 min at 30°C. The absorbance was measured three times at 340 nm using a MultiScan Ascent Microplate Reader (Thermo Fisher Scientific Inc., Waltham, MA). For fructose readings, 0.25 enzyme units (EU) of phosphoglucose isomerase was added to the extracts in each well of the plate and the absorbance was again measured at 340 nm. Subsequently, sucrose readings were obtained by adding 83 units of invertase to the extracts and the microtitration plate was incubated at 30°C for 60 min. The absorbance was measured three times at 340 nm and the results were expressed in mg carbohydrate/mg dry weight using a standard curve made of known glucose concentrations for calculation.

Experimental design and statistical analysis

The experimental design was a strip split plot with water regime as the main unit running across all blocks in strips in a randomized complete block design. Cultivars were randomly assigned in the sub unit for each whole plot in each block. The treatments water regime and cultivar were considered as fixed effect. The blocks and the block x treatment interaction were considered as random effect. Data from the control plots collected at Marianna were not significantly different between seven and fourteen days after the onset of the stress; therefore, the

data were pooled into one set of data. Data were subjected to analysis of variance and Tukey's test (α=0.05) was used to separate treatment means using JMP Pro 11 (SAS Institute, Cary, NC).

RESULTS

Stomatal Conductance

Stomatal conductance of water-stressed plants was significantly lower than the well-watered control for all cultivars at both locations (Table 1 and Fig. 1). At Marianna, stomatal conductance of plants after seven days of stress were significantly lower compared with the well-watered control (Table 1 and Fig. 1B). However, after fourteen days of stress, stomatal conductance was maintained at similar rates than after seven days of stress in all cultivars, except for DP1044 where stomatal conductance of water-stressed plants were significantly lower after fourteen days of stress in relation to seven days (Table 1 and Fig. 1B). At New Deal, all cultivars also had stomatal conductance significantly reduced by water-deficit stress (Table 1 and Fig. 1A).

Comparing the cultivars within the same water regime, differences in stomatal conductance rates were observed (Table 1 and Fig. 1). At Marianna, under well-watered control, FM2484 and DP0912 showed the highest and lowest stomatal conductance rates, respectively, among the cultivars. After seven days of stress, FM2484 still had the highest stomatal conductance among the cultivars, while the lowest rates were observed in NG1511. After fourteen days of stress, no significant differences in stomatal conductance were found among the cultivars (Table 1 and Fig. 1B). At New Deal, cultivars did not show significant differences in stomatal conductance under well-watered control. However, after seven days of stress, FM2484

and NG1511 showed the highest stomatal conductance rates and DP1044 had the lowest rates among the cultivars (Table 1 and Fig. 1A).

Leaf chlorophyll content

Different responses were found in leaf chlorophyll content among water regimes and cultivars (Table 1 and Fig. 2). At Marianna, DP0912 had leaf chlorophyll content significantly lower only after fourteen days of stress, with values after seven days similar to the well-watered control (Table 1 and Fig. 2B). In addition, ST4946 significantly increased leaf chlorophyll content at seven days of stress compared with the well-watered control, with a decrease in leaf chlorophyll content after fourteen days of stress to similar values than the control. The leaf chlorophyll content of the other cultivars were unaffected by water regimes (Table 1 and Fig. 2B). At New Deal, seven days of stress also decreased the leaf chlorophyll content of DP1044 and ST4946 in relation to the well-watered control (Table 1 and Fig. 2A). The other cultivars did not have leaf chlorophyll content affected by water regimes (Table 1 and Fig. 2A).

At Marianna, the significantly highest leaf chlorophyll content was found in FM2484, while the other cultivars did not differ among them, regardless of the water regime (Table 1 and Fig. 2B). At New Deal, cultivars had different responses in leaf chlorophyll content (Table 1 and Fig. 2A). The cultivar FM2484 showed the highest leaf chlorophyll content among the cultivars under well-watered conditions, while the lowest contents were observed in DP1044 and PHY499. After seven days of stress, the highest and lowest leaf chlorophyll content were observed in NG1511 and DP1044, respectively (Table 1 and Fig. 2A).

Carbohydrates concentration

Soluble sugars were affected by water regimes only in DP1044, FM2484, and ST4946 (Table 2). At Marianna, seven days of water-deficit stress significantly increased the soluble sugars concentrations in the plants of the cultivars cited above. However, after fourteen days of stress, soluble sugar was decreased to similar concentrations found in the well-watered control plants (Table 2). At New Deal, FM2484 had the soluble sugars reduced by seven days of water-deficit stress. Cultivars did not differ in soluble sugars concentration within the same water regime at Marianna. At New Deal, under well-watered conditions, soluble sugars concentrations were not different among the cultivars, while after seven days of water-deficit stress, DP1044 and PHY400 showed the highest concentrations of soluble sugars and FM2484 had the lowest concentration among the cultivars (Table 2).

Sucrose concentrations were increased in DP0912 after seven days of water-deficit stress and then decreased to concentrations similar to well-watered control after fourteen days of stress at Marianna (Table 2). Sucrose was unaffected by water regimes in the other cultivars at Marianna. At New Deal, all cultivars were did not have sucrose concentration affected by water regimes, except for NG1511 which had concentration increased by water-deficit stress (Table 2). Differences in sucrose concentration among the cultivars were not found in the different water regimes or locations (Table 2).

Starch concentration was decreased by seven days of water-deficit stress in DP1044 and PHY499 compared with the well-watered control at Marianna (Table 2). After fourteen days of stress, starch of these cultivars increased to concentrations similar to the control. At New Deal, no differences were observed in the cultivars between the water regimes (Table 2). Cultivars

within the same water regime were not different in starch concentration, regardless of the water regime or location (Table 2).

Total non-structural carbohydrates (NSC) was affected by water regimes only in some cultivars (Table 2 and Fig. 3). At Marianna, the cultivar PHY499 increased total NSC after seven days of stress in relation to well-watered control (Table 2 and Fig. 3B). However, after fourteen days of stress, the total NSC was decreased to concentrations similar to the control. At New Deal, seven days of water-deficit stress significantly increased total NSC of DP0912 and NG1511 compared with the well-watered control, while the other cultivars remained unaffected by water regimes (Table 2 and Fig. 3A). Cultivars did not differ in total NSC among them within the same water regime at Marianna and New Deal locations (Table 2 and Fig. 3).

DISCUSSION

As water is not supplied by means of rainfall or irrigation, plants have their physiological mechanisms impaired. The onset of drought stress leads to a cascade of events, including reduction in cell growth and division, synthesis of proteins and enzymes, and stomatal closure (Hsiao et al., 1976). Reduced stomatal conductance contributes to drought avoidance, which is a mechanism to survive a drought period (Reddy et al., 2004). Our results indicated that stomatal conductance of all cultivars was significantly reduced by water-deficit stress during the flowering stage and as the stress became more severe, some cultivars maintained stomatal conductance at rates similar to the mild stress. In accordance with our findings, other studies with cotton reported decrease of stomatal conductance of plants grown under drought conditons (Jamal et al., 2015; Loka and Oosterhuis, 2013).

It is well-known that chlorophyll degradation is one of the primary consequences of drought stress in plants (Hsiao et al., 1976). This leads to a reduction in photosynthesis with consequent decrease in photoassimilates for plant growth. Torres Neto et al. (2005) had found a strong relationship between SPAD readings and leaf chlorophyll content by means of fluorescence measurement. In our study, changes in leaf chlorophyll content of plants grown under water-deficit stress were not observed, except for DP0912 at Marianna and DP1044 at New Deal, which exhibited reduced chlorophyll content in plants subjected to fourteen and seven days of stress, respectively, in relation to the well-watered control. Research has been conducted to evaluate the relationship between SPAD readings and chlorophyll content in several species, such as wheat, potato and apple, and the authors found a weak relationship between SPAD values and extracted chlorophyll concentration (Uddling et al., 2007; Campbell et al., 1990). Even though we did not extract the leaf chlorophyll concentration, as SPAD meter measures the greenness of leaves, we speculate that these readings do not reflect the proper amount of chlorophyll present in leaves of the cotton cultivars studied, especially under drought conditions. As cited previously, SPAD meter quantifies the greenness of leaves; therefore, the higher the SPAD reading, the greener the leaf. In some species, thick and dark green leaves are associated with some level of drought tolerance (Fanizza et al., 1991). At Marianna, the cultivar FM2484 stood out clearly by showing higher SPAD readings than the other cultivars in all water regimes. This cultivar also showed remarkable SPAD readings at New Deal, with high values under well-watered conditions and after seven days of stress. The greener leaves observed in FM2484 might be associated with a level of drought tolerance of this cultivar.

Accumulation of soluble sugars is a mechanism that plants use to cope with drought stress (Chen et al., 2005). Higher concentration of soluble sugars assists in cell protection from

harmful effects of water-deficit conditions, and plants with improved accumulation of these compatible solutes are considered more tolerant to drought (Chen et al., 2002). In our study, DP1044 was the only cultivar that increased concentrations of soluble sugars, sucrose and starch after seven days of water-deficit stress, with concentrations decreasing to levels similar to the well-watered plants after fourteen days of stress. In addition, concentrations of soluble sugars were increased after seven days of water-deficit stress in the cultivars FM2484 and ST4946 at Marianna, and decreased in FM2484 at New Deal. However, after fourteen days of stress, soluble sugars was decreased to concentrations similar to the well-watered plants. Sucrose was also accumulated after seven days of water-deficit stress in NG1511 at New Deal, but after fourteen days of stress, plants had sucrose concentrations similar to the well-watered plants. The same pattern occurred for starch and total NSC in PHY499, with increased concentrations after seven days of water-deficit stress and, after fourteen days of stress, decreased starch concentrations to similar levels to well-watered plants. DP0912 and NG1511 also increased concentrations of total NSC after seven days of stress at New Deal. These results indicate that some cotton cultivars have some level of tolerance to mild drought conditions, but when the stress becomes more severe, plants are unsuccessful in accumulation of sufficient soluble sugars to help in a drought period.

CONCLUSION

Cotton cultivars did not exhibit differences in accumulation of carbohydrates under well-watered control, seven and fourteen days of water-deficit stress. FM2484 seemed to have some tolerance to drought by improving green color of leaves, and DP1044 appeared to exhibit

tolerance to mild drought conditions by accumulating high concentrations of carbohydrates in the leaves of stressed plants.

TABLES AND FIGURES

Table 1. Stomatal conductance (mmol $m^{-2}s^{-1}$) and SPAD reading of six cotton cultivars, DP0912, DP1044, FM2484, NG1511, PHY499, and ST4946, under three water regimes, control, seven days, and fourteen days of water-deficit stress, in Marianna, and two water regimes, control and seven days of water-deficit stress, in New Deal.

Location	Water regime	Cultivars					
		DP0912	DP1044	FM2484	NG1511	PHY499	ST4946
		Stomatal conductance (mmol $m^{-2}s^{-1}$)					
Marianna	Control	690.69 aB[§]	728.87 aAB	846.42 aA	741.52 aAB	773.11 aAB	748.37 aAB
	7 days	391.64 bAB	542.44 bAB	583.96 bA	303.11 bB	484.08 bAB	420.10 bAB
	14 days	326.50 bA	361.77 cA	462.66 bA	251.78 bA	402.14 bA	406.41 bA
New Deal	Control	1191.39 aA	1136.14 aA	1352.35 aA	1366.45 aA	1413.84 aA	1372.69 aA
	7 days	555.67 bAB	439.76 bB	749.56 bA	734.35 bA	544.05 bAB	555.22 bAB
		SPAD reading					
Marianna	Control	48.12 abB	47.73 aB	53.30 aA	47.70 aB	48.46 aB	46.97 bB
	7 days	49.40 aB	49.14 aB	55.86 aA	50.76 aB	49.56 aB	50.12 aB
	14 days	45.80 bB	46.32 aB	52.52 aA	48.20 aB	47.44 aB	46.84 bB
New Deal	Control	53.27 aBC	52.50 aC	56.95 aA	56.72 aAB	52.57 aC	55.50 aABC
	7 days	50.80 aBC	48.30 bC	54.62 aAB	54.90 aA	52.90 aAB	52.07 bABC

[§] Means followed by the same capital letter in the row and low case in the column within the same factor for each variable are not significantly different according to Tukey's test ($P \leq 0.05$).

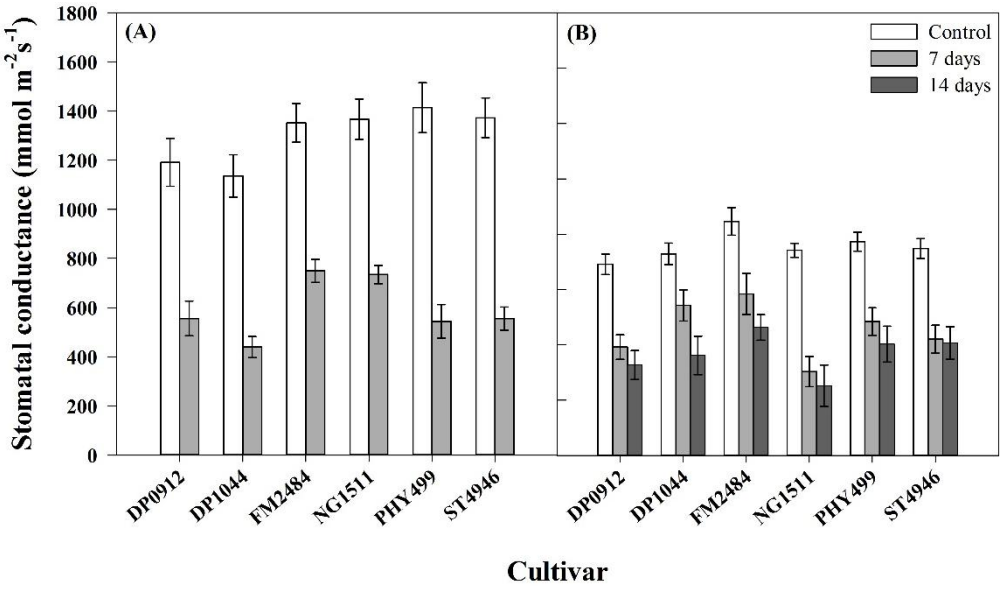

Figure 1. Stomatal conductance (mmol $m^{-2}s^{-1}$) of six cotton cultivars, D0912, DP1044, FM2484, NG1511, PHY499, and ST4946 under two water regimes in New Deal (A) and three water regimes in Marianna (B). All values are means ± standard error (n=5).

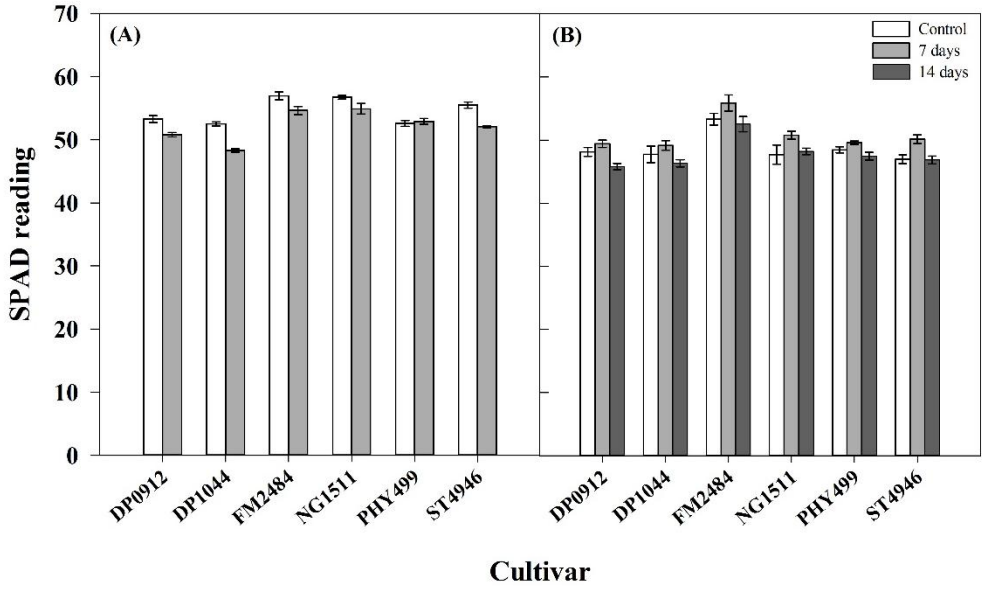

Figure 2. SPAD reading of six cotton cultivars, D0912, DP1044, FM2484, NG1511, PHY499, and ST4946 under two water regimes in New Deal (A) and three water regimes in Marianna (B). All values are means ± standard error (n=5).

Table 2. Soluble sugars, sucrose, starch, and total non-structural carbohydrates (NSC) of six cotton cultivars under three water regimes in Marianna, and two water regimes in New Deal.

Location	Water regime	Cultivars					
		DP0912	DP1044	FM2484	NG1511	PHY499	ST4946
				Soluble sugars (mg mg^{-1})			
Marianna	Control	0.0129 aA[§]	0.0155 abA	0.0141 bA	0.0143 aA	0.0128 aA	0.0107 bA
	7 days	0.0183 aA	0.0189 aA	0.0246 aA	0.0194 aA	0.0202 aA	0.0190 aA
	14 days	0.0111 aA	0.0078 bA	0.0094 bA	0.0106 aA	0.0105 aA	0.0103 bA
New Deal	Control	0.0085 aA	0.0010 aA	0.0089 aA	0.0078 aA	0.0082 aA	0.0071 aA
	7 days	0.0082 aAB	0.0096 aA	0.0061 bB	0.0086 aAB	0.0097 aA	0.0082 aAB
				Sucrose (mg mg^{-1})			
Marianna	Control	0.0164 aA	0.0010 abA	0.0110 aA	0.0149 aA	0.0127 aA	0.0157 aA
	7 days	0.0145 aA	0.0165 aA	0.0089 aA	0.0126 aA	0.0172 aA	0.0182 aA
	14 days	0.0089 aA	0.0077 bA	0.0071 aA	0.0088 aA	0.0090 aA	0.0116 aA
New Deal	Control	0.0091 aA	0.0070 aA	0.0076 aA	0.0079 bA	0.0088 aA	0.0079 aA
	7 days	0.0141 aA	0.0112 aA	0.0081 aA	0.0154 aA	0.0123 aA	0.0112 aA
				Starch (mg mg^{-1})			
Marianna	Control	0.0284 aA	0.0304 aA	0.0287 aA	0.0286 aA	0.0273 abA	0.0295 aA
	7 days	0.0271 aA	0.0239 bA	0.0292 aA	0.0251 aA	0.0261 bA	0.0267 aA
	14 days	0.0315 aA	0.0307 aA	0.0313 aA	0.0301 aA	0.0323 aA	0.0313 aA
New Deal	Control	0.0210 aA	0.0225 aA	0.0214 aA	0.0210 aA	0.0211 aA	0.0214 aA
	7 days	0.0229 aA	0.0217 aA	0.0237 aA	0.0220 aA	0.0208 aA	0.0228 aA
				Total NSC (mg mg^{-1})			
Marianna	Control	0.0577 aA	0.0558 aA	0.0538 aA	0.0579 aA	0.0528 bA	0.0559 aA
	7 days	0.0600 aA	0.0593 aA	0.0627 aA	0.0572 aA	0.0635 aA	0.0638 aA
	14 days	0.0514 aA	0.0462 aA	0.0478 aA	0.0496 aA	0.0517 bA	0.0532 aA
New Deal	Control	0.0386 bA	0.0395 aA	0.0379 aA	0.0367 bA	0.0380 aA	0.0364 aA
	7 days	0.0452 aA	0.0425 aA	0.0379 aA	0.0460 aA	0.0428 aA	0.0421 aA

[§] Means followed by the same capital letter in the row and low case in the column within the same factor for each variable are not significantly different according to Tukey's test ($P \leq 0.05$).

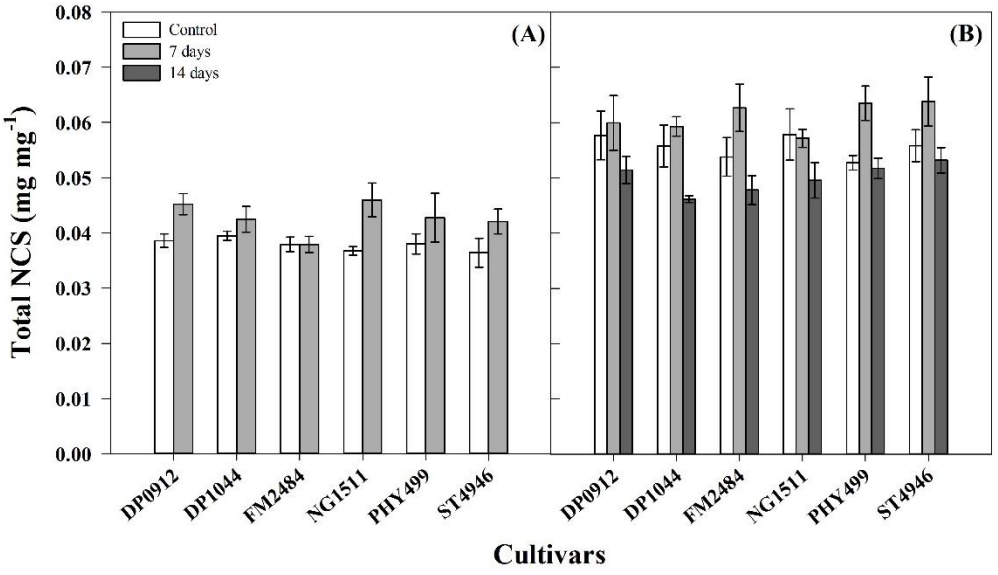

Figure 3. Total non-structural carbohydrates (NSC) (mg mg^{-1}) of six cotton cultivars, D0912, DP1044, FM2484, NG1511, PHY499, and ST4946 under two water regimes in New Deal (A) and three water regimes in Marianna (B). All values are means ± standard error (n=5).

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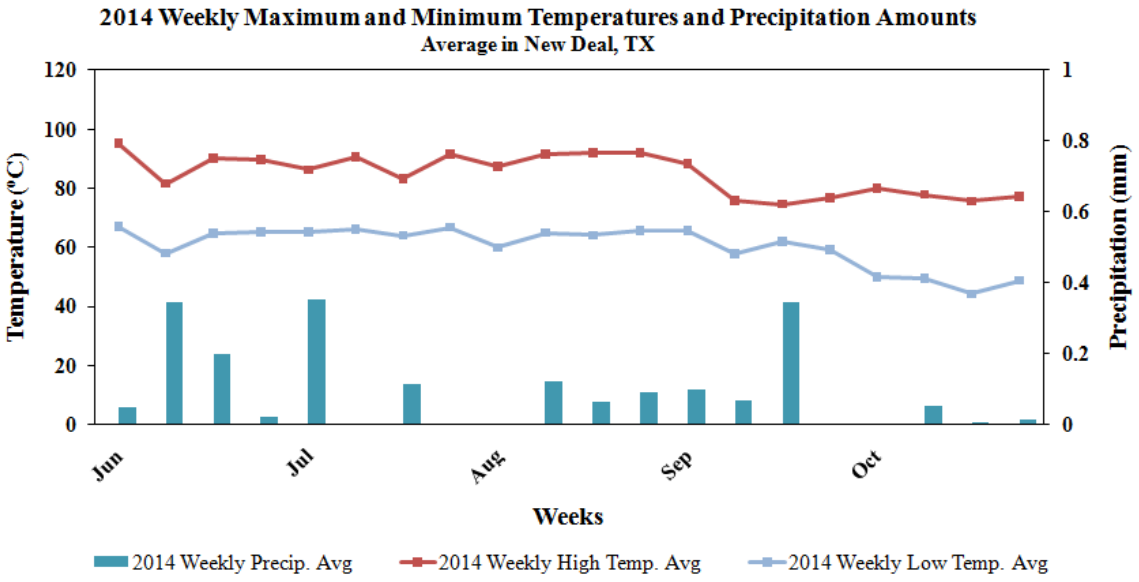
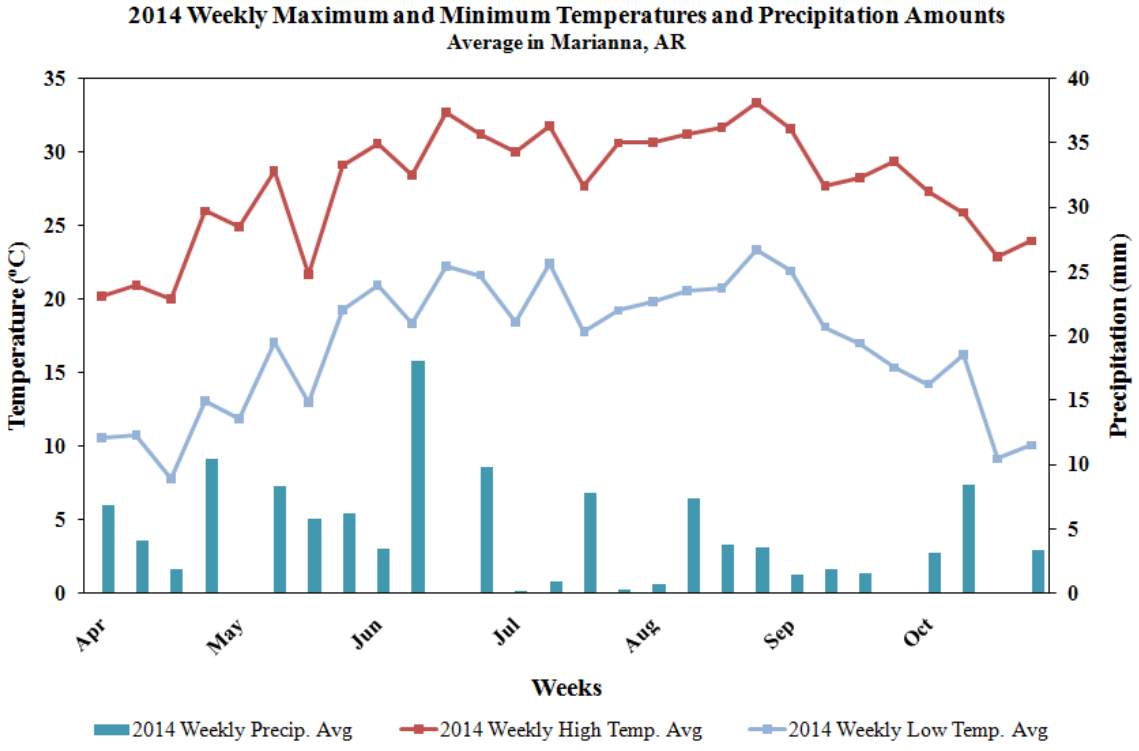
APPENDIX


2014 Weekly Maximum and Minimum Temperatures and Precipitation Amounts
Average in New Deal, TX
Temperature (°C)
Precipitation (mm)
120
100
80
60
40
20
0
1
0.8
0.6
0.4
0.2
0
Jun
Jul
Aug
Sep
Oct
Weeks
2014 Weekly Precip. Avg
2014 Weekly High Temp. Avg
2014 Weekly Low Temp. Avg


2014 Weekly Maximum and Minimum Temperatures and Precipitation Amounts
Average in Marianna, AR
Temperature (°C)
Precipitation (mm)
35
30
25
20
15
10
5
0
40
35
30
25
20
15
10
5
0
Apr
May
Jun
Jul
Aug
Sep
Oct
Weeks
2014 Weekly Precip. Avg
2014 Weekly High Temp. Avg
2014 Weekly Low Temp. Avg

VI. CARBOHYDRATE METABOLISM AND WATER POTENTIAL OF FLOWERS OF COTTON PLANTS EXPOSED TO WATER-DEFICIT STRESS DURING PEAK FLOWERING

ABSTRACT

Changes in carbohydrates metabolism of cotton leaves under water-deficit conditions have been documented to cause a reduction in energy and carbon supply by the plants with consequent decrease in plant growth. However, the relevance of flower tissues (bracts, petals, and pistils) as sources of water and carbohydrates for fruit development under water-deficit stress episodes is not completely elucidated. A field and a growth room experiment were conducted to evaluate carbohydrate metabolism in cotton flower tissues and the corresponding contribution to boll development in plants subjected to water-deficit conditions at the flowering stage. Two cotton cultivars were grown under well-watered conditions until the peak flowering stage, at which time water was withhold and measurements were taken. Stomatal conductance was measured daily in the growth room experiment throughout the stress period. Samples of white flowers from the first sympodial fruiting position and their subtending leaves were collected at the last day of the stress for measurement of water potential, carbohydrate concentration and dry matter. Results indicated that sucrose was the carbohydrate component accumulated in higher concentrations in all plant tissues. Carbohydrate metabolism of cotton leaves and flowers grown under water-deficit conditions was altered, with partitioning of carbohydrates shifted by the water-deficit stress. Subtending leaves and bracts represented the main sources, while pistils were considered the main sinks, especially for sucrose and soluble sugars. However, further studies are required

to elucidate carbohydrate metabolism in flowers of modern cotton cultivars under drought conditions during the flower development.

INTRODUCTION

Drought is one of the most important environmental factors affecting plant physiological processes and restricting crop development and yield worldwide (Chaves et al., 2009). More than one third of world's cultivated areas are provided with inadequate or lack of water supply and significant water shortage are highly likely to arise in some countries and regions in a near future (FAO, 2015).

Cotton (*Gossypium hirsutum* L.) plants that undergo a water scarcity period have their leaf stomata closed followed by a reduction in photosynthesis (Massacci et al., 2008). As drought episodes become more severe, energy and carbon supply by the plants is reduced and demand is increased, thus decreasing growth rates (Chaves, 1991). By limiting production and consumption of photoassimilates water-deficit conditions cause imbalances in the partitioning of carbon throughout the plant (Chaves, 1991). It is generally accepted that response of leaves and fruits, such as water potential and changes in carbohydrates ratios, are utilized to identify the degree of severity of drought stress in the plants (Fessender and Ehleringer, 2003). Duration and severity of the stress lead to changes in carbohydrate metabolism in the plant. Mild water-deficit stress has been reported to promote a reduction in starch and an increasing in soluble sugars in some species (DaMatta et al., 1997; Basu et al., 1999).

In cotton plants, the flowering development has been well documented as a vulnerable stage to drought conditions and it becomes less sensitive as boll development progresses (reviewed by Loka et al., 2011). Under well-watered conditions, cotton plants accumulate

photoassimilates during the daylight hours and translocate the reserves to the sinks at night (Warner and Burke, 1993). Leaves are the main source of assimilates to the developing bolls, and subtending leaves are known to contribute approximately 60% of the photoassimilates translocated to fruit set under well-irrigated conditions (Schubert et al., 1986). However, when plants experience drought conditions growth is impaired and an imbalance of carbohydrates flow occurs with 2- to 3-fold higher accumulation of sucrose in relation to well-watered plants (Timpa et al., 1986). Studies on maize (*Zea mays* L.) demonstrated that grain development overcome a water-deficit stress period due to a buffered grain water potential (ψ_w) by a reduction in leaf ψ_w (Ouattar et al., 1987). In addition to changes in plant ψ_w, drought conditions induce an accumulation of sucrose in the leaves (main source of carbohydrates) due to limited sink consumption, and an increase in starch-breakdown enzymes thus reducing starch concentrations in water-stressed leaves (Zeeman et al., 2004; Quick et al., 1989). Liu et al. (2004) reported an imbalance in carbohydrates status of soybean (*Glycine max* L.) plants grown under drought stress, contributing to pod abortion. Also, research on wheat (*Triticum aestivum* L.) suggested that disturbance in carbohydrates availability in the reproductive organ may cause pollen sterility (Dorion et al., 1996; Saini et al., 1984). Loka and Oosterhuis (2013), studying changes in carbohydrates metabolism of cotton plants subjected to water-deficit stress, demonstrated that cotton leaves under water-deficit stress had glucose concentrations increased and maintained sucrose concentrations similar to well-watered plants, while flower pistils of stressed plants had sucrose concentrations increased and glucose and fructose remained unaffected by the water-deficit stress. The authors suggested that the opposite responses between leaves and pistils might be attributed to tissue specific regulation of sucrose breakdown enzymes, with invertase as up-regulated in the leaves and down-regulated in the fruiting tissues. However, the importance of

flower tissues (bracts, petals, and pistils) as sources of water and carbohydrates for ovary development into a boll during water-deficit stress episodes is poorly understood. Therefore, the objective of this study was to evaluate the distribution of carbohydrates components in cotton flower tissues and their contribution to boll development in plants under water-deficit stress during the flowering stage.

MATERIALS AND METHODS

Experiments were conducted in the field at the Arkansas Agricultural Research and Extension Center (AAREC) (N 36°05'48", W 94°10'41") and in a growth chamber at the Altheimer Laboratory of the University of Arkansas in Fayetteville, AR in 2014.

The field was arranged in a strip block design composed of four treatments and five replications and the growth room was arranged in a randomized complete block design with four treatments and ten replications. Treatments consisted of two cotton (*Gossypium hirsutum* L.) cultivars, DP 0912 B2RF (Delta and Pine Land, Monsanto Company, St. Louis, MO) and PHY 499 WRF (PhytoGen, Dow AgroSciences, Indianapolis, IN), and two water regimes, well-watered control and water-deficit stress imposed at peak flowering (approximately 70 days after planting).

In the field, seeds were sown on May 20th at a seeding rate of 10 seeds m^{-1} in a Captina silt loam (fine-silty, siliceous, active, mesic Typic Fragiudult) soil. A total of 20 plots, each composed of 4 rows, 15.3 m in length with a row spacing of 0.96 m, were used for the experiment with measurements and samples being taken in the two middle rows of each plot. To further characterize site conditions, the average weekly maximum and minimum temperatures, and precipitation of each location throughout the season are presented in Appendix.

Fertilization was performed according to soil tests prior to planting and recommended rates for cotton. Herbicide and pesticide applications were also applied according to University of Arkansas Cooperative Extension Service recommendations. Mepiquat chloride was applied as needed to control vegetative growth and all plots received identical applications.

The whole field was irrigated with furrow system according to University of Arkansas Cooperative Extension Service recommendations until peak flowering. When plants reached this stage on August 19th, water was withheld from the water-stress treatment for ten days. White flowers from the first sympodial fruiting position and their subtending leaves were collected for measurements of water potential, carbohydrate concentration, and dry matter.

In the growth room, seeds were planted in 2-L pots filled with Sunshine potting media (Sun Gro Horticulture Distribution Inc., Bellevue, WA) in a large growth chamber (Model PW36, Conviron, Winnipeg, Canada). Temperatures of 32/24°C (day/night), 14h photoperiod, 60% relative humidity, and a typical diurnal pattern of the photosynthetically active radiation (PAR) with the highest PAR (850 µmol m^{-2} s^{-1}) between 10:00 am and 02:00 pm were maintained in the growth chamber. The plants were watered once daily with half-strength Hoagland's nutrient solution (Hoagland and Arnon, 1933) to maintain adequate moisture until peak flowering approximately 70 days after planting. At this stage, water stress was imposed by withholding water from the water-stressed plants until stomatal conductance (g_s) reached approximately 20 mmol $m^{-2}s^{-1}$ indicating that the stomata were virtually closed. Well-watered control plants received an optimum quantity of water throughout the duration of the experiment. Once the water-stressed plants reached the required stress, white flowers from the first fruiting position and their subtending leaves were collected for determination of water potential, carbohydrate concentrations, and dry matter.

Stomatal conductance

Stomatal conductance was measured daily starting at the first day of the stress (peak flowering) until the plants reached approximately 20 mmol $m^{-2}s^{-1}$ using a steady-state leaf porometer (SC-1 Leaf Porometer, Decagon, Pullman, Washington, USA) on the abaxial surface of fourth uppermost fully expanded main-stem leaves. Measurements were taken between 12:00 pm to 02:00 pm with two readings per leaf (which were averaged) due to the small surface area of the cuvette (6.25 mm^2). The results were expressed as mmol H_2O $m^{-2}s^{-1}$.

Water potential

Discs (10 mm diameter) of petals from white flowers in the first sympodial fruiting position and subtending leaves were excised from cotton plants from 11:00 am to 1:00 pm for determination of water potential (Ψ_w). Samples were measured with screen-caged thermocouple psychrometers (model 74 series, J.R.D. Merrill Specialty Equipment, Logan, UT) equipped with stainless steel sample chambers using the technique described by Oosterhuis (2003). Water potentials were determined with samples being equilibrated in waterbath at 25°C for 4 hours and readings taken using a micro-voltmeter and chart recorder. Water potential results were expressed in MPa.

Carbohydrates concentration

Soluble carbohydrate content was measured according to Hendrix (1993) with modifications. White flowers from the first sympodial fruiting position were separated into bracts, petals, and pistil. Flower components and subtending leaves were oven dried for 5 days at 50°C and then ground for further analysis. Forty mg of the ground tissue were extracted 3 times

with 80% aqueous ethanol (800 mL ethanol / L) and the samples were centrifuged after each extraction at 5000 rpm and then the fractions were pooled. Active charcoal was added to the fractions to remove substances that could alter the carbohydrate measurements and the samples were centrifuged at 3500 rpm. The supernatant was immediately stored in -80°C for determination of sucrose and hexose (fructose and glucose) and the residue was stored in fridge for 20 h at 4°C for determination of starch.

For starch determination, 0.5 mL of potassium hydroxide (KOH) was added in each sample and placed in water bath at 100°C for 1 h. After the samples were cooled down, the pH was adjusted to 7 using 1M acetic acid. Then 50 µL of TRIS buffer and 100 µL a-amylase were added and samples were placed in water bath at 85°C for 30 min. After this, 0.5 mL amyloglucosidase was added and samples were incubated in water bath at 55°C for 60 min. Enzyme reaction was stopped by heating the samples in a thermoblock at 100°C for 5 min. Deionized water was added to the samples to reach final volume of 1.5 mL and samples were centrifuged in 10000 rpm for 10 min. For starch readings, 20 µL of the sample, 10 µL of water and 100 µL of the Glucose Assay Reagent were pipetted in each well of a microtitration plate. Plate was incubated at 30°C for 15 min and absorbance was read at 340 nm.

For soluble sugars, the glucose (HK) assay kit from Sigma (Sigma Chemical Company, St. Louis, MO) was used. A 20 µL aliquot of each extract was pipetted into a well of a microtitration plate and the plate was incubated at 50°C for 40 min to evaporate ethanol. For glucose readings, 10 µL of water was added to each well along with 100 µL of glucose assay reagent and the plate was incubated again for 15 min at 30°C. The absorbance was measured three times at 340 nm using a MultiScan Ascent Microplate Reader (Thermo Fisher Scientific Inc., Waltham, MA). For fructose readings, 0.25 enzyme units (EU) of phosphoglucose

isomerase was added to the extracts in each well of the plate and the absorbance was again measured at 340 nm. Subsequently, sucrose readings were obtained by adding 83 units of invertase to the extracts and the microtitration plate was incubated at 30°C for 60 min. The absorbance was measured three times at 340 nm and the results were expressed in mg carbohydrate/mg dry weight using a standard curve made of known glucose concentrations for calculation.

Dry matter

White flowers from the first sympodial fruiting position, which were separated into bracts, petals, and pistil were oven dried for 7 days at 60°C and then weighted for determination of dry matter. Results were expressed in grams.

Statistical analysis

The experimental design in the field was a strip block design with water regime as the main unit running across all blocks in strips in a randomized complete block design. Cultivars were randomly assigned in the sub unit for each whole plot in each block. Growth room experiment was a randomized complete block design with one plant as one experimental unit. The treatments water regime and cultivar were considered as fixed effect. The blocks and the block x treatment interaction were considered as random effect. As similar trends and no significant differences were observed for dry matter in flower tissues between the field and growth room experiments, the results were pooled and the means were taken. Data of the field and growth room experiments were subjected to analysis of variance and Tukey's test (α=0.05)

was used to separate treatment combination mean performance using JMP Pro 11 (SAS Institute, Cary, NC).

RESULTS

Stomatal Conductance

Leaf stomatal conductance was significantly lower in water-stressed plants compared with the well-watered control after two days of the onset of water-deficit stress (Fig. 1). Stomatal conductance of the two cultivars remarkably decreased two days after water was withheld in the water-deficit plants, reaching the desired stomatal rate of approximately 20 mmol $m^{-2}s^{-1}$ three days after the onset of the stress.

Cultivars did not show significant differences in stomatal conductance within the same water regime for either water-deficit or well-watered conditions (Fig. 1).

Water Potential

Water potential (ψ_w) was measured in the leaves and petals of cotton plants. Similar trends were observed in the field and growth room conditions with lower (more negative) water potential in leaves and petals of water-stressed plants compared with the well-watered control for both DP0912 and PHY499 (Figs. 2 and 3).

In the field, leaf water potential of water-stressed plants was 43% and 47% more negative than the well-watered control for DP0912 and PHY499, respectively (Fig. 2A). Petal water potential of plants under water-deficit stress was 49% and 41% lower in relation to well-watered control for DP0912 and PHY499, respectively (Fig. 2B).

In the growth room, similar trends to the field were observed, but the percentages in difference of water potential between water-stressed and well-watered plants were bigger in the leaves and smaller in the petals (Figs 2 and 3). Leaf water potential of water-stressed plants was 45% and 53% more negative than the well-watered plants for DP0912 and PHY499, respectively (Fig. 3A), while petal water potential was 44% and 37% lower in stressed plants than well-watered control for DP0912 and PHY499, respectively (Fig. 3B).

Carbohydrates Concentration

Total non-structural carbohydrates (NSC) was significantly decreased by water-deficit conditions in the field, regardless of the cultivar (Fig. 4). In the growth room, total NSC was unaffected by water-deficit stress for both cultivars; however, water-deficit conditions reduced (numerically) the total NSC of DP0912 and increased (numerically) the total NSC available in PHY499.

In the field, under well-watered conditions, the proportion of total NSC available in the subtending leaves was 23% and 26% and pistils was 15% and 16% for DP0912 and PHY499, respectively. However, when the plants are subjected to water-deficit stress, the proportion of total NSC is altered, with 19% and 21% of the total NSC available in the subtending leaves of DP0912 and PHY499, respectively, and 18% in the pistils for both cultivars.

In the growth room, the cultivars showed different responses than the field, with the proportion of total NSC available of 20% in the subtending leaves of well-watered plants for both cultivars and 19% and 22% in pistils of DP0912 and PHY499, respectively. The proportion of total NSC was slightly altered in water-stressed plants, with 21% and 17% in subtending leaves and 22% and 23% in the pistils.

In the field experiment, water-deficit stress caused a significant decrease in soluble sugars and sucrose concentrations in the subtending leaves of DP0912, while starch concentration remained unaffected (Fig. 5a). For PHY499, concentrations of soluble sugars, sucrose and starch in the subtending leaves were decreased by water-deficit conditions (Fig. 5b). Bracts of water-stressed plants of DP0912 had the sucrose concentration increased compared with the well-watered control, and soluble sugars and starch concentrations were not significantly affected by water regimes (Fig. 5c). On the other hand, carbohydrate concentrations were different for PHY499, with significantly lower starch concentration in water-stressed bracts in relation to the control and no effect of water regimes on soluble sugars and sucrose concentrations (Fig. 5d). Concentrations of soluble sugars, sucrose and starch in petals of flowers from both cultivars were not affected by water-deficit stress (Fig. 5e,f). Concentrations of sucrose and starch in the pistil were significantly increased by water-deficit stress in DP0912, while soluble sugars were unaffected by water regimes (Fig. 5g). For PHY499, starch was the only carbohydrate component affected by water-deficit stress, with significantly lower concentration in the pistil of water-stressed plants (Fig. 5h).

The cotton plants cultivated in the growth room showed a different response regarding carbohydrates concentrations in comparison with the field experiment (Figs. 5 and 6). Concentrations of soluble sugars, sucrose and starch in the subtending leaves were unaffected by water regimes for DP0912 and PHY499 (Fig. 6a,b). Soluble sugars and starch concentrations were decreased in bracts of plants grown under water-deficit stress for DP0912 (Fig. 6c). On the other hand, water-deficit stress significantly increased concentrations of soluble sugars and sucrose in bracts of PHY499 (Fig. 6d). Petals of stressed plants in DP0912 had only soluble sugars affected by water regimes, with lower concentrations in the water-deficit treatment (Fig.

6e), while concentrations of carbohydrates units of petals remained unaffected by water regime in PHY499 (Fig. 6f). Concentrations of carbohydrates in the pistil were not significantly affected by water regimes in DP0912 (Fig. 6g); however, sucrose concentrations significantly increase in the pistil of water-stressed plants compared with well-watered plants (Fig. 6h).

Dry matter of flower tissues

Water-deficit stress imposed at peak flowering decreased dry matter of flower tissues (Fig. 7). Compared with the flower tissues developed under well-watered conditions, significant decreases were observed in bracts, petals, and pistils of water-stressed plants of the two cultivars grown under field and growth chamber conditions. In DP0912, the decrease in dry matter was approximately 19%, 29% and 33% for bracts, petals, and pistils, respectively. Similarly, in PHY499, the decrease in dry matter was approximately 19%, 27% and 30% for bracts, petals, and pistils, respectively.

Differences in dry matter of bracts, petals, and pistils were not noticed between the cultivars within the same water regime, both under well-watered and water-stressed conditions (Fig. 7).

DISCUSSION

Stomatal closure is well-reported as a plant response to limited water supply to reduce water loss by the crop (Massacci et al., 2008; Hsiao, 1973). Our research demonstrated significant reductions in leaf stomatal conductance of cotton plants subjected to water-deficit stress compared with the well-watered control, regardless of the cultivar. Several studies have documented decreases in leaf stomatal conductance of cotton plants grown under drought

conditions (Jamal et al., 2015; Bielorai and Hopmans, 1975). In a study with cotton plants, Loka and Oosterhuis (2013) also indicated significant reduction in leaf stomatal conductance as water supply in the soil was decreased.

In addition to stomatal closure, leaf water potential is considered as an indicator of plant water balance (Karamanos, 2003). In our study, we measured water potential in leaves and petals of white flowers. A reduction in water potential (more negative values) of both tissues was observed, demonstrating that the plants subjected to water-deficit conditions responded to the stress by lowering water potential in vegetative and reproductive tissues. Leaf water potential was more negative than petal water potential for the two cultivars studied. A study on maize has indicated that grain water potential of plants under drought conditions was buffered by a reduction in leaf water potential (Ouattar et al., 1987). We speculate that cotton plants respond similarly with a more remarkable reduction in leaf water potential in order to buffer petal water potential, thus preventing water loss in reproductive units.

As a consequence of low leaf water potential and reduced stomatal conductance in both cultivars, photosynthesis is also expected to be reduced (Massacci et al, 2008). The limitation of CO_2 diffusion from atmosphere to carboxylation sites has an effect on carbohydrates metabolism in the plant tissues. Water-deficit stress reduced the total NSC available in both cultivars studied in the field. Subtending leaves contribute approximately 60% of all photoassimilates used in development of fruiting sites (Schubert et al., 1986). Water limitation significantly reduced the total NSC available in subtending leaves of cotton plants compared with the well-watered control.

Growth is associated with carbon availability in the plants, therefore the sensitivity of plant growth is related to remobilization and consume of carbohydrates under water-deficit stress

(Pedroso et al., 2014). Water-stressed plants had the growth of flower tissues (bracts, petals and pistils) significantly reduced. Studies have been documented that plants from numerous species grown under limited water conditions have inhibition of shoot growth and stimulation of root growth to improve water uptake (Galvez et al., 2011; Pace et al., 1999). Even though we did not evaluate the root system of the cotton cultivars, we speculate that carbohydrates were translocated to the root system to maintain (or improve) growth as an acclimation response to drought.

Carbohydrate metabolism is documented to be directly involved with plant growth (Smith and Stitt, 2007), and as plant growth was affected by water-deficit stress, alterations in carbohydrate concentration are expected to occur. The distribution of carbohydrates among the cotton plant tissues was different between the cultivars and also the water regimes. Carbohydrate metabolism in subtending leaves was either reduced in water-stressed plants (field) or unaffected by water stress (growth room). Differences in plant response grown under field and growth room conditions might be explained by the difference in time, duration and severity of water-deficit stress between the two growth conditions. In the bracts, carbohydrates were either increased or unaffected by water-deficit stress, except for soluble sugars in DP0912 in the growth room experiment where the concentrations were decreased by the stress. Bracts are considered photosynthetic organs and our results support the statement that the importance of bracts as assimilatory tissues in cotton plants increases during adverse environmental conditions, such as drought, with higher contribution in carbohydrates assimilation when leaf photosynthesis is reduced (Wullschleger et al., 1990). Petals were not affected by water-deficit conditions, except for the soluble sugars in DP0912 grown under growth room conditions, with lower concentrations in water-stressed plants. The buffered water potential in the petals may explain

the carbohydrate metabolism in stressed plants similar to levels found in well-watered control. Under water deficit, pistils are important sinks of carbohydrates (especially sucrose) as the pistils increased sucrose concentrations under water-deficit conditions (statistically and numerically). One possible explanation is that the ovaries (part of the pistil) grow into bolls responsible for seeds production and consequently crop yield, thus the plants would ensure reproduction even with lower plant growth.

CONCLUSION

Sucrose was the carbohydrate accumulated in higher concentrations in all plant tissues. Water-deficit stress alters carbohydrate metabolism in cotton plants. The stress promoted a shift in the carbohydrate partitioning, with subtending leaves and bracts as main sources and pistils as main sinks, mainly for sucrose and soluble sugars. Further research is needed for complete elucidation of carbohydrate metabolism in flowers of modern cotton cultivars under drought conditions during the flower development.

FIGURES

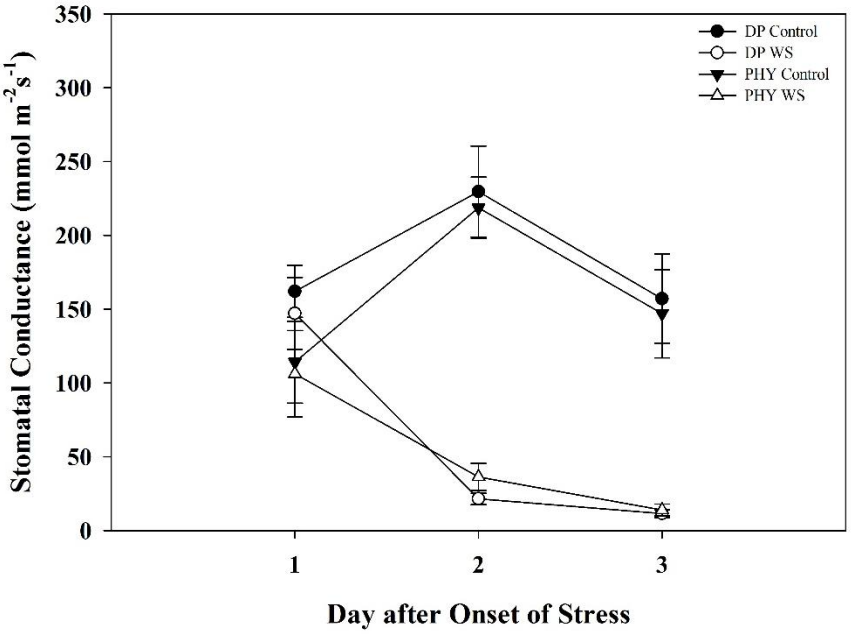


Figure 1. Temporal changes in stomatal conductance (mmol $m^{-2}s^{-1}$) of two cotton cultivars, DP 0912 B2RF (DP) and PHY 499 WRF (PHY) grown under two water regimes, well-watered control (closed symbols) and water-deficit stress (open symbols) under growth room conditions. Values are means ± standard error (n=10).

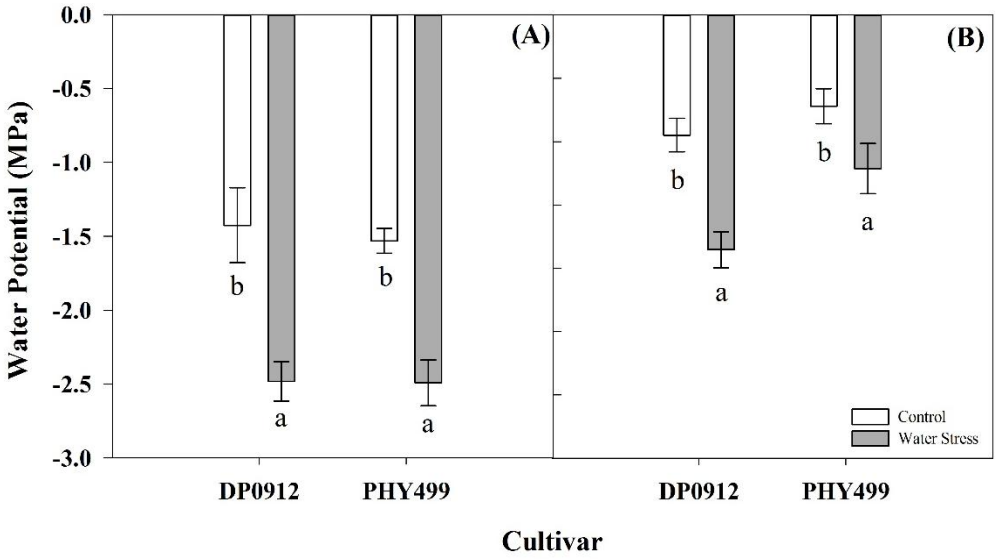


Figure 2. Water potential (MPa) of leaves (A) and petals (B) of two cotton cultivars, DP 0912 B2RF and PHY 499 WRF under two water regimes, well-watered control and water-deficit stress grown under field conditions. All values are means ± standard error (n=5). Different letters indicate significant difference between water regimes within the same cultivar according to Tukey's test ($P \leq 0.05$).

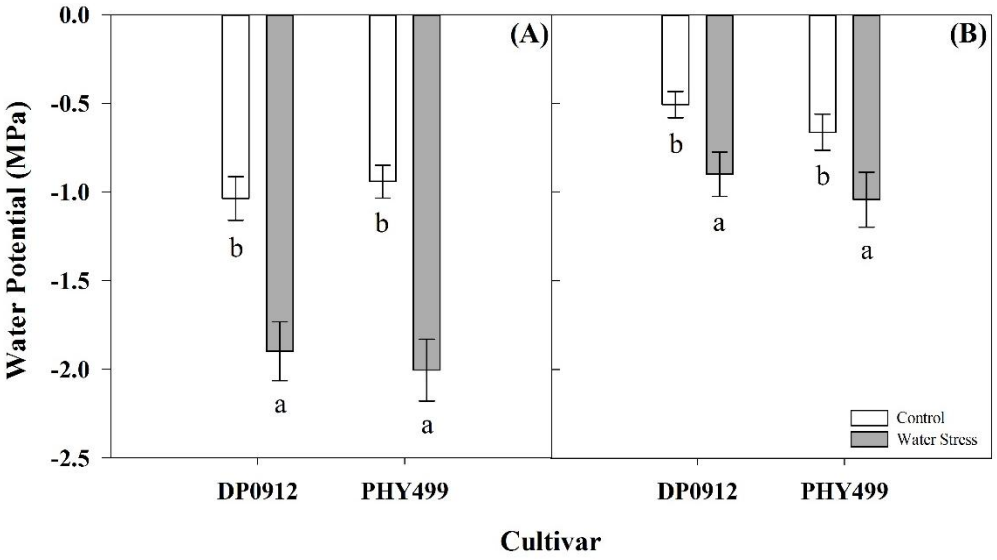


Figure 3. Water potential (MPa) of leaves (A) and petals (B) of two cotton cultivars, DP 0912 B2RF and PHY 499 WRF under two water regimes, well-watered control and water-deficit stress grown under growth room conditions. All values are means ± standard error (n=10). Different letters indicate significant difference between water regimes within the same cultivar according to Tukey's test ($P \leq 0.05$).

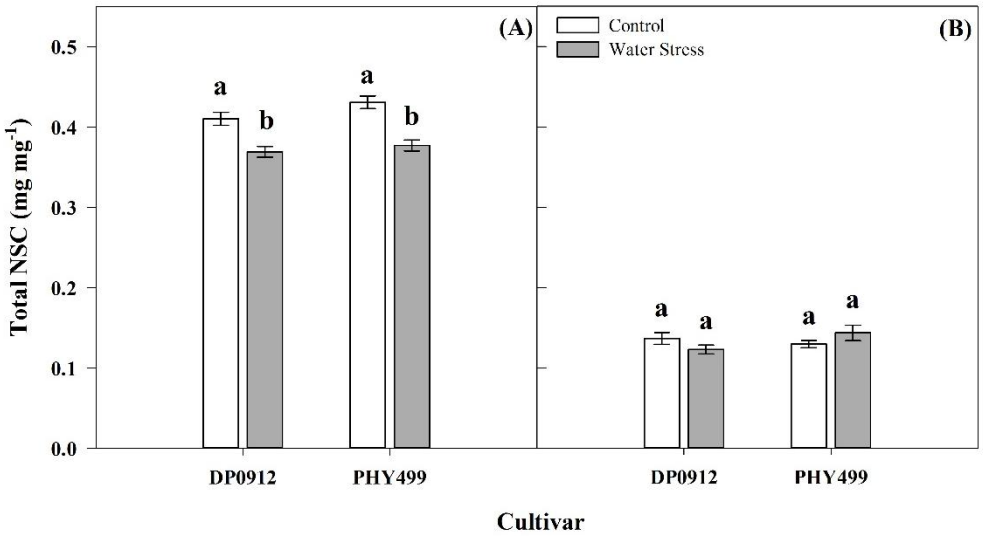


Figure 4. Total non-structural carbohydrates (NSC) of two cotton cultivars, DP 0912 B2RF and PHY 499 WRF, under two water regimes, well-watered control and water-deficit stress grown under field (A) and growth room (B) conditions. All values are means ± standard error (n=5 in the field and n=10 in the growth room). Different letters indicate significant difference between water regimes within the same cultivar according to Tukey's test ($P \leq 0.05$).

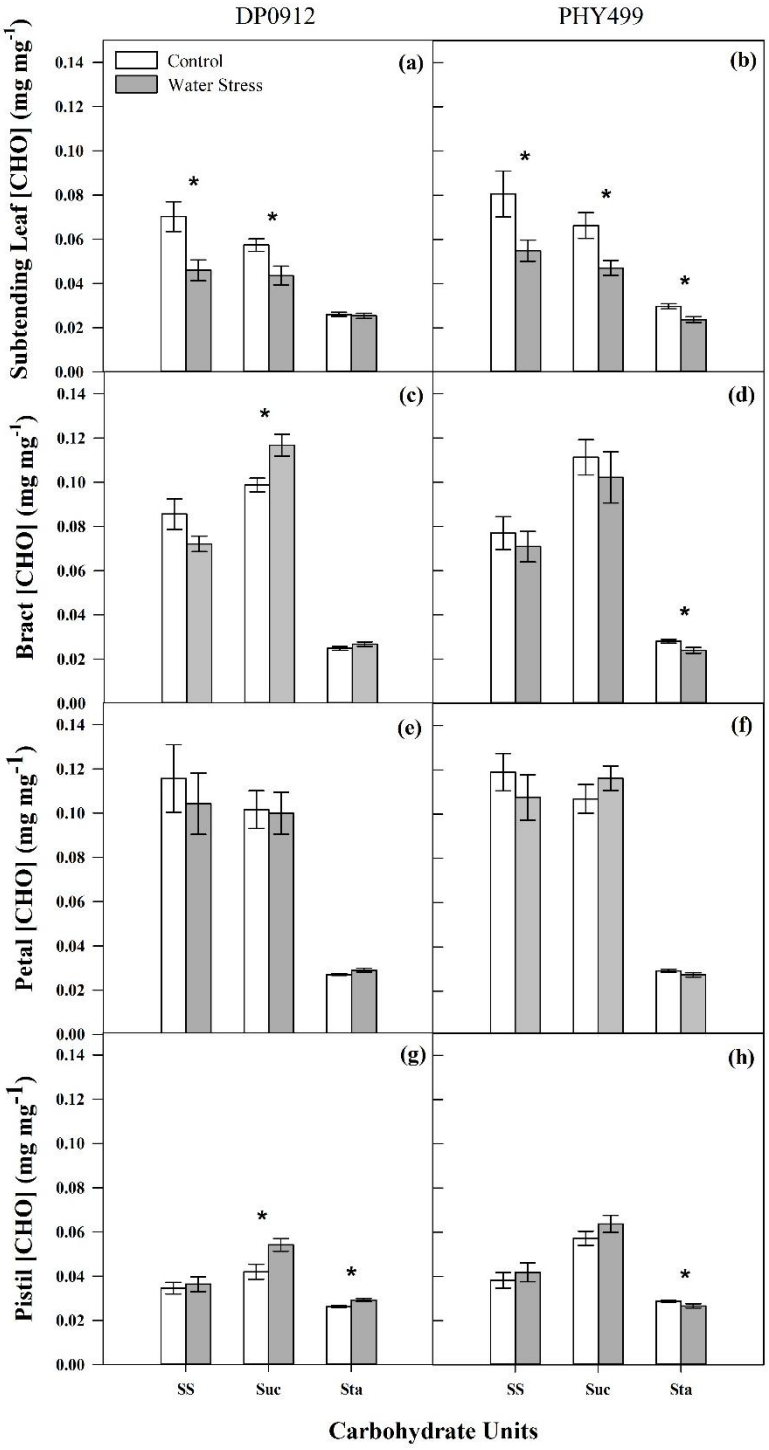


Figure 5. Concentrations of soluble sugars (SS), sucrose (Suc), and starch (Sta) in subtending leaves (a and b), bracts (c and d), petals (e and f), and pistils (g and h) of two cotton cultivars, DP 0912 B2RF (a, c, e, and g) and PHY 499 WRF (b, d, f, and h) under two water regimes, well-watered control and water-deficit stress grown under field conditions. All values are means ± standard errors (n=5). Asterisks indicate significant differences between water regimes within the same carbohydrate unit according to Tukey's test ($P \leq 0.05$).

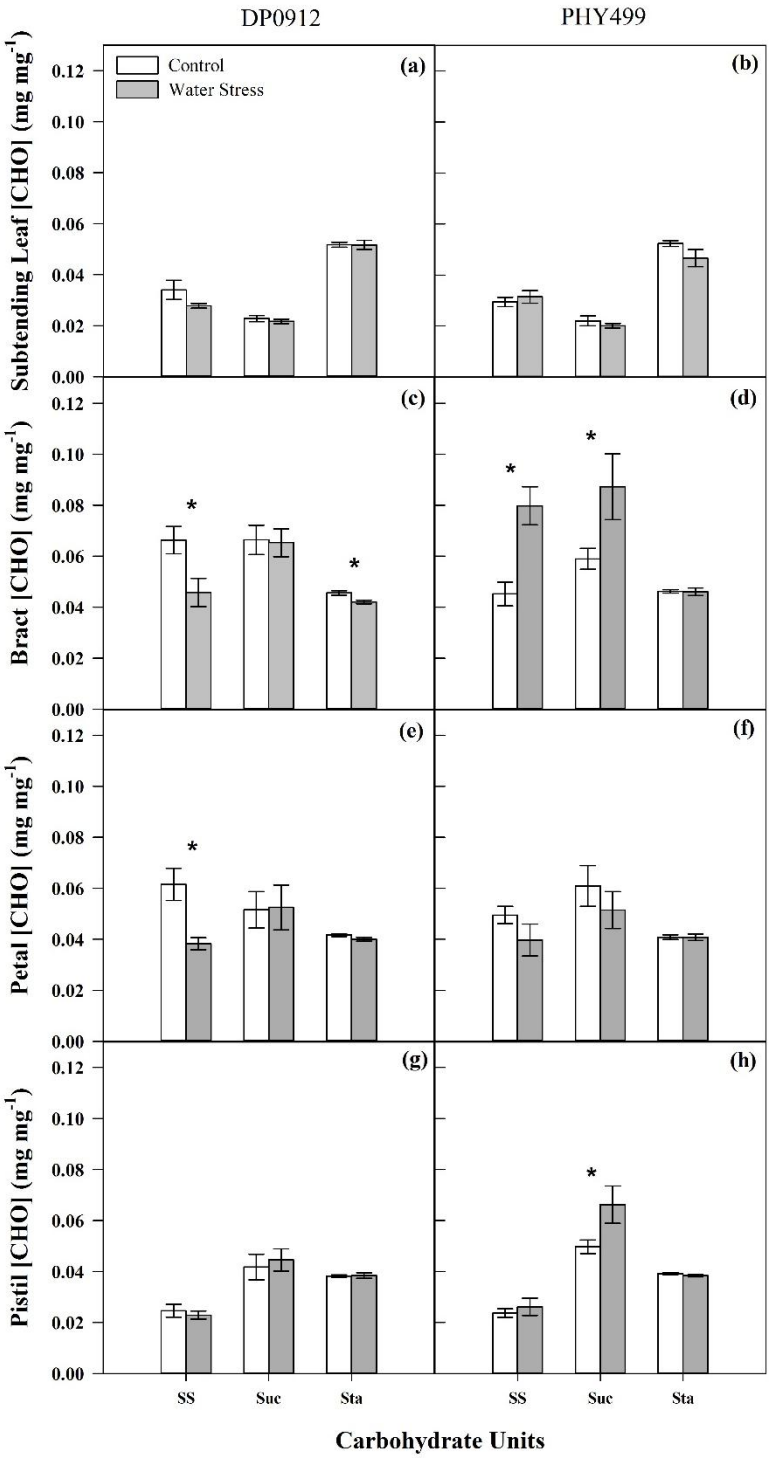


Figure 6. Concentrations of soluble sugars (SS), sucrose (Suc), and starch (Sta) in subtending leaves (a and b), bracts (c and d), petals (e and f), and pistils (g and h) of two cotton cultivars, DP 0912 B2RF (a, c, e, and g) and PHY 499 WRF (b, d, f, and h) under two water regimes, well-watered control and water-deficit stress grown under growth room conditions. All values are means ± standard errors (n=10). Asterisks indicate significant differences between water regimes within the same carbohydrate unit according to Tukey's test ($P \leq 0.05$).

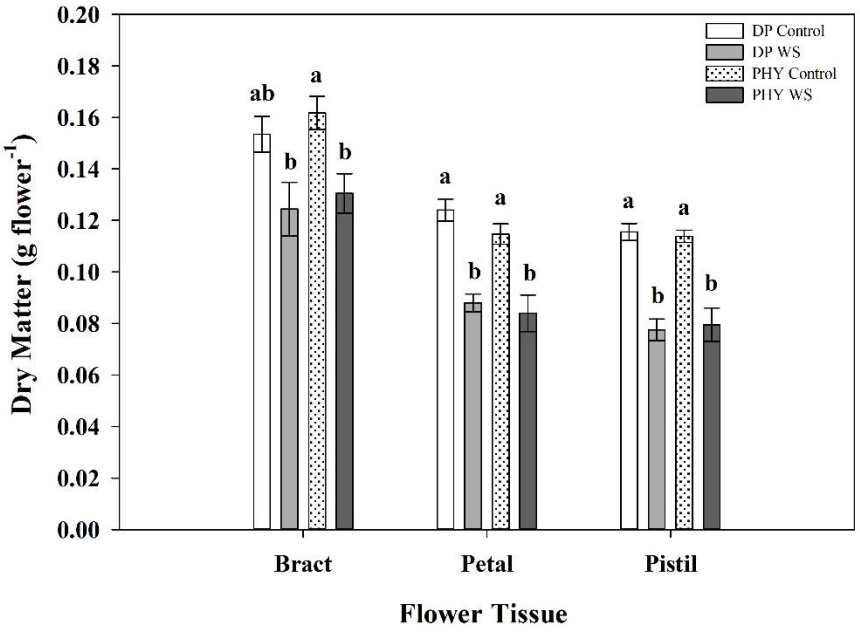


Figure 7. Dry matter (g flower^{-1}) of bracts, petals, and pistils of two cotton cultivars, DP 0912 B2RF (DP) and PHY 499 WRF (PHY) under two water regimes, well-watered control (Control) and water-deficit stress (WS). All values are means ± standard error. Bars within the same flower tissue sharing the same letter are not significantly different according to Tukey's test ($P \leq 0.05$).

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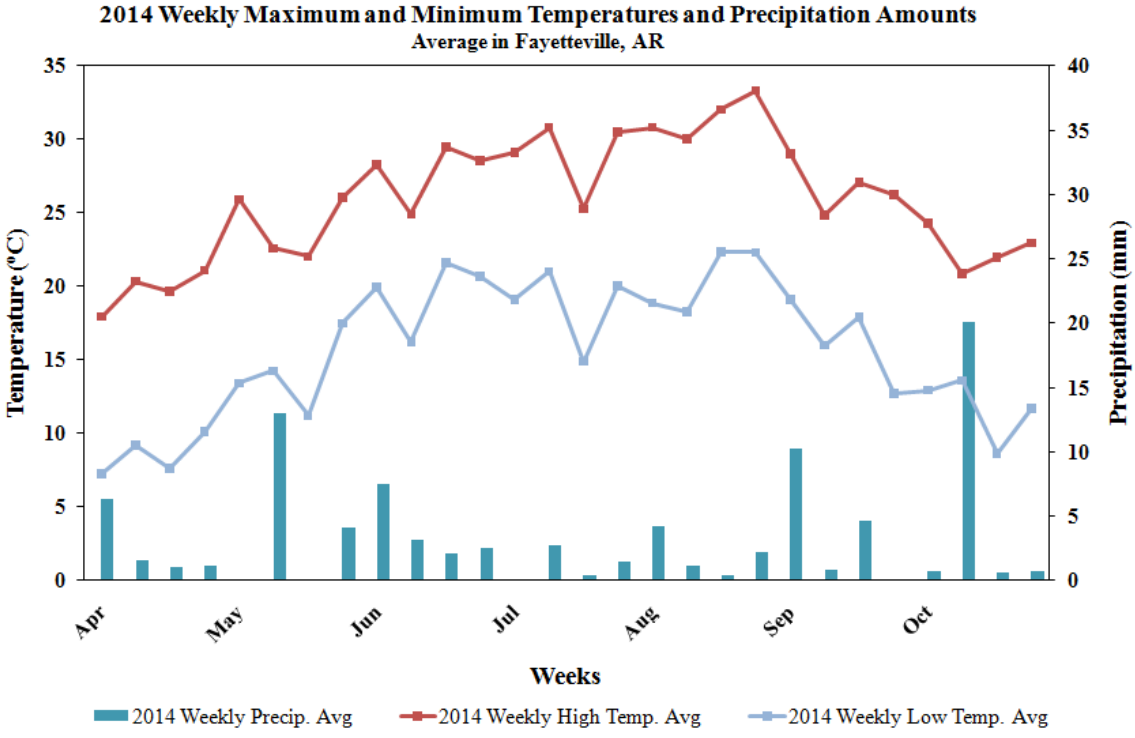
APPENDIX


2014 Weekly Maximum and Minimum Temperatures and Precipitation Amounts
Average in Fayetteville, AR
Temperature (°C)
Precipitation (mm)
35
30
25
20
15
10
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0
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20
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0
Apr
May
Jun
Jul
Aug
Sep
Oct
Weeks
2014 Weekly Precip. Avg
2014 Weekly High Temp. Avg
2014 Weekly Low Temp. Avg

VII. OSMOTIC ADJUSTMENT IN LEAVES AND OVARIES FROM WHITE FLOWERS OF COTTON CULTIVARS UNDER WATER-DEFICIT STRESS AT FLOWERING STAGE

ABSTRACT

Previous investigations have demonstrated that osmotic adjustment is a mechanism used by several species to tolerate a period of drought conditions, but no information has been reported on osmotic adjustment in vegetative and reproductive units of modern cotton cultivars (*Gossypium hirsutum* L.). We hypothesized that osmotic adjustment would be a mechanism used by modern cotton cultivars and would be higher in the leaves than ovaries due to more negative osmotic potential in the vegetative units and also there would exist variation in osmotic adjustment between the cotton cultivars. A field experiment was conducted in Lubbock, TX and growth room experiments were performed at the University of Arkansas in Fayetteville, AR. Two and four diverse cotton cultivars were planted in the field and growth room experiments, respectively. Plants were submitted to water-deficit stress during flowering stage for ten days (field) and four days (growth room). Stomatal conductance was measured daily in the growth room experiment throughout the stress period. At the end of the stress, samples of ovaries from white flowers in the first position in the main stem and their subtending leaves were collected for osmotic potential and proline concentration analysis. Cottonseed yield was obtained at the end of season in the field experiment. Results indicated that osmotic adjustment through proline accumulation and changes in osmotic potential is used by the cotton cultivars studied to tolerate a drought period. Leaves are suggested to be more sensitive to water-deficit stress than ovaries, showing higher osmotic adjustment. Cultivars varied in tolerance to water-deficit stress, with

PHY499 and Siokra L23 indicating the highest level of tolerance due to higher osmotic adjustment in the leaves and ovaries, respectively.

INTRODUCTION

Water is essential for numerous plant physiological functions including plant nutrient transport, chemical and enzymatic reactions, cell expansion, and transpiration (Hsiao, 1973). Plants under water-deficit stress have their biochemical and physiological processes altered affecting plant growth (Gardner et al., 1983; Kramer, 1980). In general, a plant is defined as drought stressed when its water potential and turgor decrease inhibiting regular plant processes (Hsiao, 1973). Crop yields have decreased under water-deficit stress, and current world climatic trends changes might further increase the severity of the problem (DaMatta et al., 2010).

Cotton is considered to be a relatively drought tolerant crop since it originates from perennial wild types that grow in areas with scarce precipitation (Lee, 1984). However, yield losses in modern cultivars under water-deficit conditions is due to domestication of cotton and its cultivation as an annual crop instead of perennial, since most of the drought tolerant characteristics are associated with the indeterminate type of growth (Quisenberry et al., 1981).

Under drought stress, osmotic adjustment occurs in plant cells through accumulation of compatible solutes in the cytosol (Xiong and Zhu, 2002). The term "compatible solutes" includes amino acids and their derivates, betaine, polyamines, proteins, soluble carbohydrates and polyols. All these compatible solutes are highly soluble and do not interfere with cell metabolism even in high concentrations (Bray et al., 2000). In most plants, osmotic adjustment through the accumulation of osmolytes has the function of reducing the osmotic potential of the cell and thus increasing the gradient for water flux inside the cell in order to maintain cell turgor and growth

(Fumis et al., 2002; Nikolaeva et al., 2010; Mafakheri et al., 2010). The turgor maintenance through osmotic adjustment contributes to continued physiological processes, such as stomatal conductance and photosynthesis (Blum, 1996).

Proline is one of the most common compatible osmolytes in plants under drought stress (Bray et al., 2000). Proline concentrations are maintained by combining the synthesis and catabolism of the amino acid (Bray et al., 2000). Proline accumulation may represent a regulatory mechanism of water loss by reducing the cell water potential (Fumis et al., 2002); however, it also serves as a biochemical marker of metabolic changes caused by stress (Lima et al., 2004). As in most plants, leaf water potential (ψ_w) is reduced under drought conditions, but cotton has the ability to osmotically adjust and maintain a higher leaf osmotic potential (ψ_l) (Oosterhuis and Wullscheleger, 1987; Turner et al., 1986; Nepomuceno et al., 1998).

Osmotic adjustment has been reported as a useful mechanism of tolerance for plants grown under water-deficit stress, which could be improved in crops through plant breeding, marker-assisted selection or genetic manipulation, to create drought-tolerant cultivars (Serraj and Sinclair, 2002). The development of drought-tolerant cultivars would assist in sustaining physiological processes of plants under drought conditions. Identification of physiological traits conferring tolerance to drought, including osmotic adjustment, and the use of them as selection criteria into breeding programs would serve as new tools for plant improvement to increase yield stability (Tuberosa and Salvi, 2006).

Morgan (1984) reported that osmotic adjustment in plants differs according to factors, such as genotype and organ type and age. Oosterhuis and Wullschleger (1987) found higher osmotic adjustment in roots than leaves of cotton plants. Studies on osmotic adjustment have also been performed with different crops (Borgo et al., 2015; Marechaux et al., 2015; Jamal et

al., 2015); however, little information is known on modern cotton cultivars. Therefore, we hypothesize that osmotic adjustment will be higher in the leaves than ovaries due to more negative osmotic potential in the vegetative organ and also variation between the cotton cultivars will exist.

The objectives of this study were to evaluate the osmotic adjustment in leaves and ovaries through accumulation of proline in the cells of cotton plants grown under water-deficit stress during the flowering stage, and identify differences in osmotic adjustment among the cultivars.

MATERIALS AND METHODS

A field experiment was conducted at the New Deal Research Farm from Texas Tech University in New Deal, TX (N 33°44'13", W 101°43'58") in 2013. The experimental design was a strip block design composed of four treatments and four replications. Treatments consisted of two cotton (*Gossypium hirsutum* L.) cultivars, ST 5288B2F (Stoneville, Bayer CropScience, Lubbock, TX) and PHY 499 WRF (PhytoGen, Dow AgroSciences, Indianapolis, IN), and two water regimes, well-watered control and water-deficit stress imposed at peak flowering (approximately 75 d after planting). Seeds were sown on May 22nd at a seeding rate of approximately 11 seeds m^{-1} in a Pullman clay loam (fine, mixed, superactive, thermic Torrertic Paleustolls) soil. To further characterize site conditions, the average weekly maximum and minimum temperatures, and precipitation throughout the season are presented in Appendix. Fertilization was performed with accordance to soil tests prior to planting and with respect to recommended rates for cotton. Herbicide and pesticide applications were also applied according to Texas A&M AgriLife extension recommendations. Mepiquat chloride was applied as needed

to control vegetative growth and all plots received identical applications. A total of 16 plots, each composed of 4 rows, were used for the experiment.

The whole field was irrigated with subsurface drip system according to Texas Tech University New Deal farm's weather station until peak flowering for ideal growth and adequate soil moisture. When plants reached peak flowering in July 26th, water was withheld from the water-stress treatment for ten days. Optimum quantity of water was applied in the well-watered control plots throughout the growing season. After ten days of water-deficit stress, the whole field (well-watered control and water-stressed plots) was re-watered in order for the plant cells to reach full turgor. Samples for osmotic potential and proline concentration were collected 12 h after the field was re-watered. Ovaries of white flowers from the first position on the main stem and their subtending leaves were collected from the two middle rows of each plot for measurements of osmotic potential and proline concentration. Cottonseed yield was obtained at the end of the growing season.

Along with the field experiment, a growth room experiment was conducted in 2013 (and repeated in 2014) at the Altheimer Laboratory, University of Arkansas in Fayetteville, AR. The experimental design was a randomized complete block design with six treatments and five replications. Treatments consisted of four cotton genotypes, DP 0912 B2RF (Delta and Pine Land, Monsanto Company, St. Louis, MO), PHY 499 WRF (PhytoGen, Dow AgroSciences, Indianapolis, IN), ST 5288 B2F (Stoneville, Bayer CropScience, Lubbock, TX) and Siokra L23 (drought tolerant Australian cultivar), and two water regimes, a well-watered control and a water-deficit stress.

Cotton genotypes were planted in 2-L pots filled with Sunshine potting media (Sun Gro Horticulture Distribution Inc., Bellevue, WA) in a large growth chamber (Model PW36,

Conviron, Winnipeg, Canada). Temperatures of 32/24°C (day/night), 14h photoperiod, 60% relative humidity, and a typical diurnal pattern of the photosynthetically active radiation (PAR) with the highest PAR (850 µmol m^{-2} s^{-1}) between 10:00 am and 2:00 pm were maintained in the growth chamber. The plants were watered once daily with half-strength Hoagland's nutrient solution (Hoagland and Arnon, 1950) to maintain adequate moisture until one week after the appearance of white flowers in the first position on the main stem (flowering stage). At this stage, water-deficit stress was imposed by withholding water from the water-stressed plants until stomatal conductance (g_s) reached approximately 20 mmol $m^{-2}s^{-1}$ indicating that the stomatal mechanism was virtually ceased. Well-watered control plants received an optimum quantity of water throughout the duration of the experiment. Once the water-stressed plants reached the required stress, all the pots were re-watered in order for the plant cells to reach full turgor. Samples for osmotic potential and proline concentration were collected 12 h after the plants were re-watered.

Stomatal conductance

Stomatal conductance was measured daily between 12:00pm to 2:00pm in the growth room experiment starting at the first day of the stress (flowering stage) until the plants reached approximately 20 mmol $m^{-2}s^{-1}$ using a steady-state leaf porometer (SC - Leaf Porometer, Decagon, Pullman, Washington, USA) on the abaxial surface of fourth uppermost fully expanded main-stem leaves with two readings per leaf (which were averaged) due to the small surface area of the cuvette (6.25 mm^2). The results were expressed as mmol H_2O $m^{-2}s^{-1}$.

Proline concentration

Proline concentration was measured in samples from both the field and growth room experiments according to methodology described by Bates et al. (1973). Ovaries from white flowers in the first position in the main stem and their subtending leaves were collected for proline analysis. Ground tissue samples (50 mg) were placed in tubes with 4 mL of sulfosalicylic acid and centrifuged in 3000 rpm for 10 min. For the colorimetric test, 1.0 mL aliquots of the crude extract along with 1.0 mL of acid-ninhydrin and 1.0 mL glacial acetic acid were pipetted in tubes. Samples were placed in water-bath at 100°C for 1 hour for color development and reaction was terminated in ice bath. Absorbance readings were performed in a UV-visible spectrophotometer (UV-1700 PharmaSpec, Shimadzu, Kyoto, Japan) at a wavelength of 520 nm. Proline concentration was determined using a standard curve as reference and calculated on a fresh weight basis (Bates et al., 1973). Results were expressed as μmol g^{-1} DM.

Osmotic potential

Ovaries from white flowers in the first position in the main stem and subtending leaf discs (10 mm diameter) from both the field and growth room experiments were collected for determination of osmotic potential (Ψ_s). Samples were measured with screen-caged thermocouple psychrometers (model 74 series, J.R.D. Merrill Specialty Equipment, Logan, UT) equipped with stainless steel sample chambers using the technique described by Oosterhuis (2003). Osmotic potentials were determined after the psychrometer-chambers were frozen in liquid N for 5 minutes, thawed at room temperature, and then allowed to equilibrate in a waterbath at 25°C for 4 hours. Readings were made using a micro-voltmeter (J. R. D. Merrill Specialty Equipment, Inc, Logan, UT) and chart recorder and results were expressed as MPa.

Cottonseed yield

Cottonseed yield was determined by mechanically harvesting the two middle rows of each plot of the field experiment. Data were expressed as kg ha^{-1}.

Statistical analysis

The experimental design in the field was a strip block design with water regime as the main unit running across all blocks in strips in a randomized complete block design. Cultivars were randomly assigned in the sub unit for each whole plot in each block. The treatments water regime and cultivar were considered as fixed effect. The blocks and the block x treatment interaction were considered as random effect. In the growth room experiment, as similar trends were observed for stomatal conductance, proline concentration, and osmotic potential in the two years of experiment, the results were pooled and the means were taken. Data of the field and growth room experiments were subjected to analysis of variance and Tukey's test (α=0.05) was used to separate treatment combination mean performance using JMP Pro 11 (SAS Institute, Cary, NC).

RESULTS

Stomatal conductance

In the growth room experiment, the stomatal conductance of each cultivar was significantly reduced by water-deficit stress (Fig 1.). Stomatal conductance rates in the stressed plants were approximately 74%, 91%, 85%, and 84% lower than the well-watered control for DP0912, PHY499, Siokra L23, and ST5288, respectively.

Significant differences in stomatal conductance were not found among the cultivars subjected to water-deficit stress; however, cultivars under well-watered control showed differences in stomatal conductance. For instance, PHY499 had the highest rates and DP0912 and Siokra L23 had the lowest rates (Fig. 1).

Proline concentration

Both in the field and growth room experiments, significantly higher concentrations of proline were accumulated in the leaves of all cultivars grown under water-deficit stress compared with the well-watered control plants (Figs. 2 and 3A). In the field, proline concentration in the leaves was 28% and 33% higher in the stressed plants compared with the well-watered plants of PHY499 and ST5288, respectively. In the growth room, the proline concentration in the leaves was 70%, 76%, 48%, and 40% higher in the stressed plants compared with the well-watered plants of DP0912, PHY499, Siokra L23, and ST5288, respectively (Fig. 3A).

Cultivars under water-deficit conditions showed differences in proline accumulation in the leaves. The highest concentration was found in PHY499 in the field and growth room experiments (Figs. 2 and 3A) and lowest concentration in Siokra L23 in the growth room (Fig. 3A). Well-watered plants also had significant differences in proline concentration in the leaves among the cultivars. In the field experiment, PHY499 accumulated higher proline concentration than ST5288 (Fig. 2), and in the growth room experiment, ST5288 showed the highest proline accumulation and the other cultivars did not differ among them (Fig. 3A).

Accumulation of proline in the ovaries followed a different trend than the leaves. PHY499 and ST5288 accumulated significantly higher concentrations of proline in the ovaries of water-stressed plants than the well-watered plants both in the field and growth room experiments

(Figs. 2 and 3B). In the field, concentrations of proline in the stressed plants of PHY499 and ST5288 were 85% and 83% higher than the well-watered control, respectively, while in the growth room, concentrations in the stressed plants of PHY499 and ST5288 were 25% and 27% higher than the well-watered control, respectively. Siokra L23 also accumulated 59% higher concentrations in the ovaries of stressed plants; however, DP0912 did not show significant difference in proline concentration between the water regimes (Fig. 3B).

Cultivars under both well-watered and water-stressed conditions did not differ in proline concentration in the ovaries between them in the field experiment (Fig. 2). However, in the growth room, a comparison of the cultivars under well-watered conditions showed that Siokra L23 and DP0912 had the highest and lowest proline concentration in the ovaries, respectively (Fig. 3B). Under water-stressed conditions, Siokra L23 had the highest concentration, and the other cultivars did not differ among them.

Osmotic potential

Leaf osmotic potential (Ψ_l) was significantly affected by water-deficit stress only in ST5288 in the field experiment and DP0912 in the growth room, with 31% and 26% higher Ψ_l in plants under water-deficit stress than the well-watered control of ST5288 and DP0912, respectively (Figs. 4 and 5A). In the field, PHY499 and ST5288 did not show significant differences in the leaf osmotic potential between them both under well-watered control and water-deficit stress (Fig. 4). In the growth room, leaf osmotic potential was statistically similar among the cultivars under well-watered control (Fig. 5A). However, under water-deficit stress, leaf osmotic potential differed among cultivars (Fig. 5A). DP0912 and PHY499 showed leaf osmotic potential significantly lower (more negative) than Siokra L23 and ST5288.

In the field experiment, ovary osmotic potential (Ψ_o) was significantly affected by water regimes only in the PHY499, with plants under water-deficit stress showing Ψ_o 75% more negative than the well-watered plants (Fig. 4). Osmotic potential in the ovaries was significantly similar between the two cultivars grown under water-deficit stress, while, under well-watered control, ST5288 indicated osmotic potential more negative than PHY499. In the growth room, water-deficit stress lowered significantly the ovary osmotic potential of DP0912 and ST5288, with Ψ_o 52% and 38% more negative in the stressed plants than the control of DP0912 and ST5288, respectively (Fig. 5B). Differences in osmotic potential among cultivars within the same water regime were also found in the grown room experiment. Under well-watered control, Siokra L23 had the more negative ovary osmotic potential and DP0912 and ST5288 had the less negative Ψ_o (Fig. 5B). Under water-deficit stress, ST5288 showed the less negative Ψ_o and the other cultivars showed more negative Ψ_o and did not differ among them (Fig. 5B).

Cottonseed yield

Cottonseed yield was significantly affected by water regimes in PHY499 and ST5288, with lower yield in the plants subjected to water-deficit stress during flowering (Fig. 6). Cultivars did not show significant differences between them under both well-watered and water-stressed conditions.

DISCUSSION

It is known that water-deficit conditions trigger stomatal closure in plants (Osakabe et al., 2014). As water availability lowers, plants close stomata to avoid loss of inner water to atmosphere. Our results indicate that plants of all cultivars reduced significantly the stomatal

conductance after four days of water-deficit stress. In a study with two cotton genotypes, Jamal et al. (2015) also demonstrated that stomatal conductance of drought-stressed plants is significantly reduced as uptake of water by the roots is decreased.

In addition to stomatal closure, plants developed several mechanisms to tolerate water-deficit stress, and osmotic adjustment is one of the main mechanisms (Chaves et al., 2003). Osmotic adjustment plays a role in tolerance to drought periods through accumulation of compatible solutes (low molecular weight solutes and inorganic ions) that reduce the osmotic potential of the cells therefore reducing water loss (Gagneul et al., 2007). This mechanism delays cells dehydration under drought conditions as it maintains cell turgor and continued physiological processes of the plants (Chen and Jiang, 2010) (Fig. 7).

Proline is an amino acid present in plant cells and has been reported as one of the main compatible solutes in osmotic adjustment in plants under drought stress (Gubis et al., 2007; Poustini et al., 2007). Its accumulation at high concentrations in plant cells is considered non-toxic and beneficial to plants as it protects plants from cell damage due to low water availability (Zhang et al., 2002). Results of our study indicated that proline was highly accumulated in leaves and ovaries of cotton plants showing that both vegetative and reproductive units are relatively sensitive to drought conditions and osmotic adjustment through proline accumulation is a mechanism used to tolerate low water availability. In support of our observations, Parida et al. (2008) and Ronde et al. (2000) reported that proline concentration was remarkably increased in leaves of cotton genotypes subjected to drought conditions. However, from our knowledge, there is no information on accumulation of proline in ovaries of cotton flowers grown under drought conditions. Significantly different concentrations of proline were found in leaves and ovaries comparing the cotton cultivars. Under water-deficit stress, PHY499 was the cultivar with higher

accumulation of proline in the leaves and Siokra L23 accumulated remarkably higher concentrations of proline in the ovaries. Parida et al. (2008) and Ronde et al. (2000) also demonstrated differences in proline accumulation among cotton genotypes, indicating that there is variation in drought tolerance through proline accumulation in the cells.

The accumulation of compatible solutes in the cells, such as proline, increases cell osmolarity driving influx of water or reducing efflux in the cells (Gomes et al., 2010). This strategy provides the turgor essential for cell expansion and ongoing physiological mechanisms. In our research, osmotic potential was generally more negative in the leaves than ovaries in all cultivars. In the field experiment, ST5288 indicated significantly lower osmotic potential in the leaves of stressed plants than the control and no differences between water regimes in the ovaries. However, in the growth room experiment, the opposite occurred with lower osmotic potential in the ovaries of stressed plants and no differences in the leaves of both water regimes. PHY99 also showed contrasting responses in ovary osmotic potential comparing the field and growth room experiments. In the field, osmotic potential of ovaries of stressed plants was significantly lower than the control, and in the growth room no significant differences were found in ovary osmotic potential between the two water regimes. We speculate that the contrasting response when field and growth room experiments are compared might be due to differences in light intensity between field and growth room, as well as differences in duration and severity of water-deficit stress between the two growth conditions. In addition, DP0912 demonstrated osmotic potential more negative in leaves and ovaries of stressed plants, while Siokra L23 had similar osmotic potential in both water regimes in the leaves and ovaries.

Differences in osmotic potential in the leaves and ovaries among the cultivars within the same water regime were found in the two growth conditions. In accordance with our results,

Jamal et al. (2015) showed differences in osmotic potential and proline accumulation in leaves between cotton cultivars. The differences in osmotic potential along with variation in proline accumulation are suggested to be indication of levels of drought tolerance in the cotton cultivars. PHY499 demonstrated a great tolerance to drought by accumulating high concentration of proline in the leaves and leaf osmotic potential more negative than the other cultivars. Siokra L23 also showed high tolerance to drought due to remarkable proline accumulation in the ovaries and ovary osmotic potential more negative than ST5288 (and significantly similar to the other cultivars).

Even though the cultivars grown in the field varied in osmotic adjustment levels, cottonseed yield was reduced by the water-deficit stress regardless of the cultivar. In addition, PHY499 and ST5288 indicated significantly similar cottonseed yields within the same water regime (well-watered control and water-deficit stress).

CONCLUSION

Osmotic adjustment through proline accumulation and changes in osmotic potential is a mechanism used by cotton plants to tolerate drought stress. Leaves are more sensitive to water-deficit stress than ovaries, thus with higher osmotic adjustment. Cultivars varied in tolerance to drought, with PHY499 and Siokra L23 indicating the highest level of tolerance due to higher osmotic adjustment in the leaves and ovaries, respectively.

FIGURES

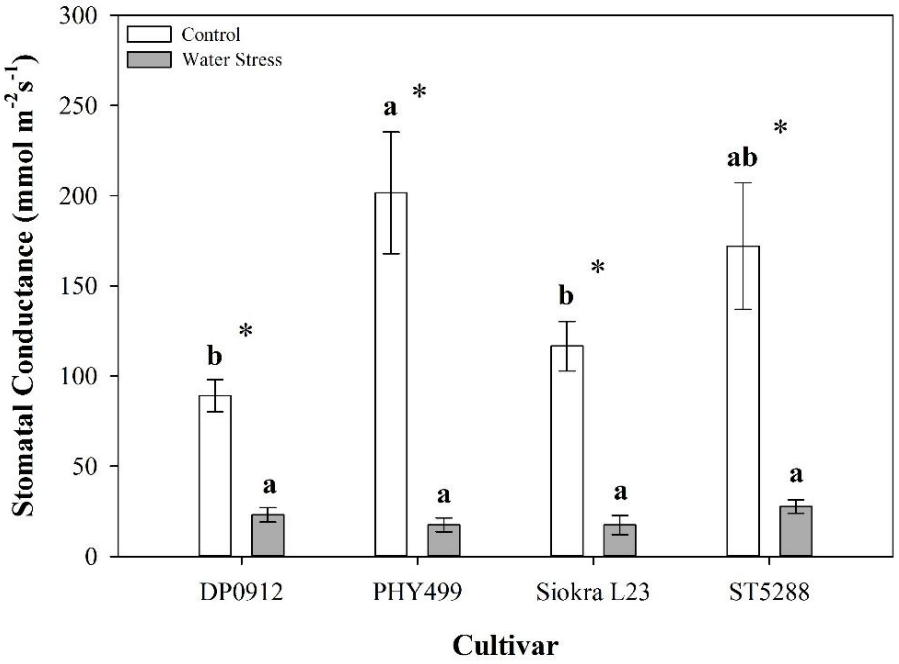

Figure 1. Stomatal conductance (mmol $m^{-2}s^{-1}$) of four cotton cultivars, DP 0912 B2RF, PHY 499 WRF, Siokra L23, and ST 5288B2F under two water regimes, well-watered control and water-deficit stress under growth room conditions. All values are means ± standard error (n=5). Asterisks indicate significant difference between water regimes within the same cultivar. Cultivars within the same water regime not sharing a common letter are significantly different ($P \leq 0.05$).

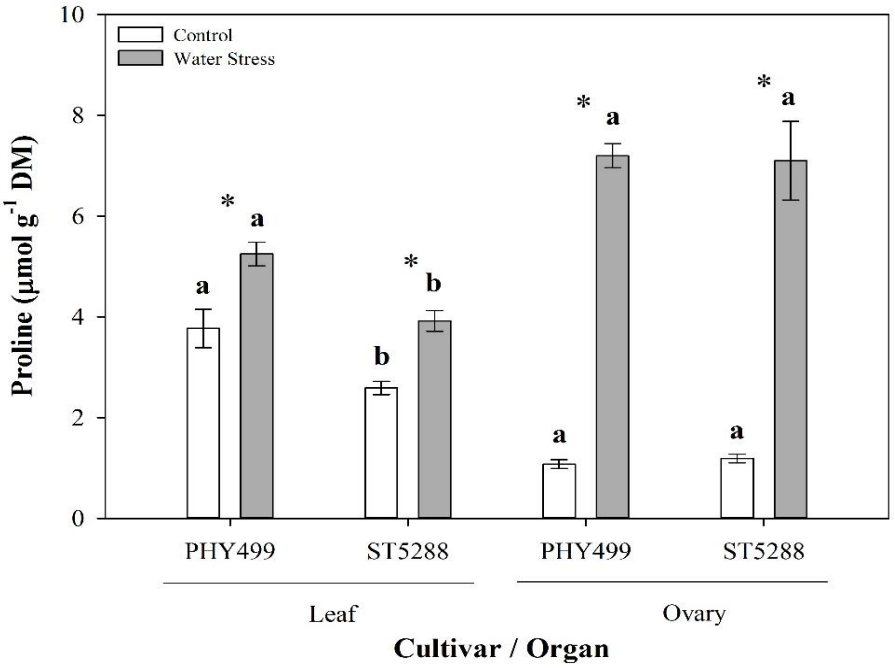

Figure 2. Proline concentration (µmol g^{-1} DM) of leaves and ovaries of two cotton cultivars, PHY499 and ST5288 under two water regimes, well-watered control and water-deficit stress grown under field conditions. All values are means ± standard error (n=4). Asterisks indicate significant difference between water regimes within the same cultivar. Cultivars within the same water regime not sharing a common letter are significantly different ($P \leq 0.05$).

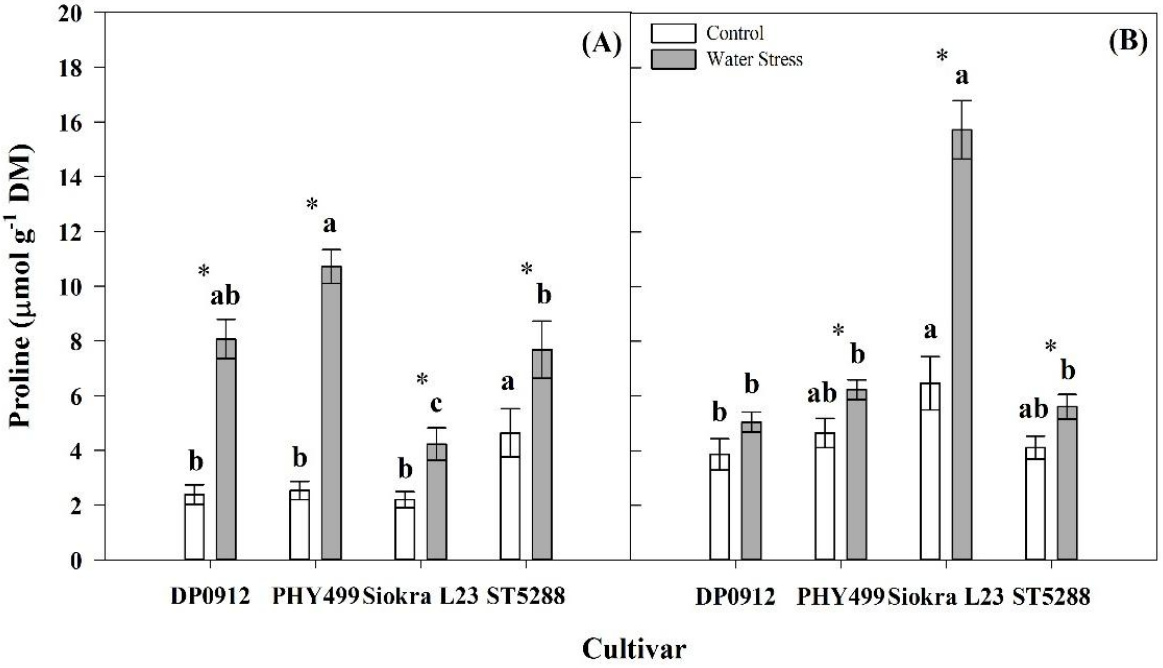

Figure 3. Proline concentration (µmol g^{-1} DM) of four cotton cultivars, DP0912, PHY499, Siokra L23, and ST5288 under two water regimes, well-watered control and water-deficit stress in the leaves (A) and ovaries (B) of plants grown under growth room conditions. All values are means ± standard error (n=5). Asterisks indicate significant difference between water regimes within the same cultivar. Cultivars within the same water regime not sharing a common letter are significantly different ($P \leq 0.05$).

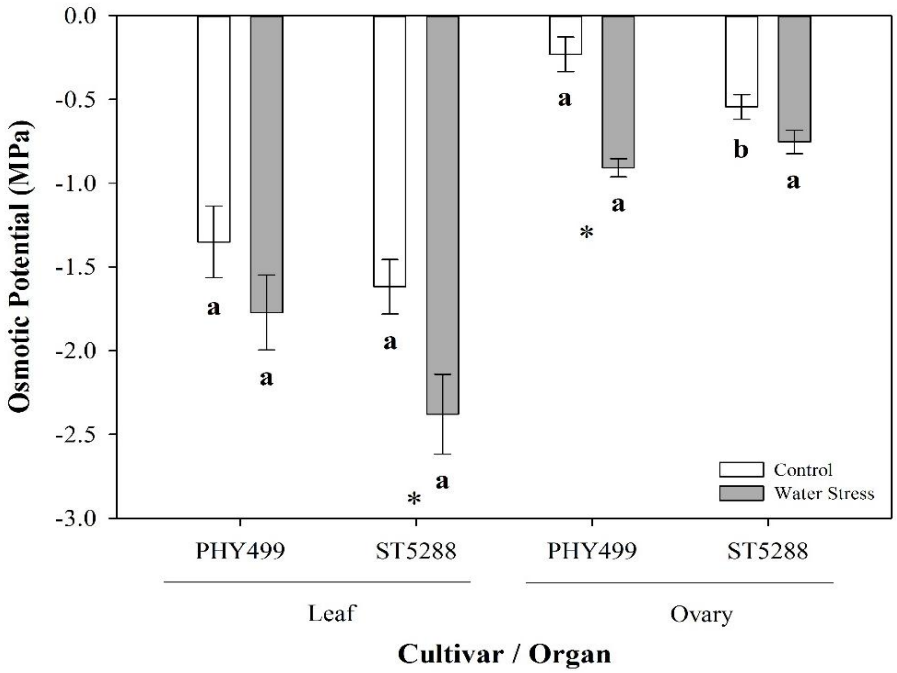

Figure 4. Osmotic potential (MPa) of leaves and ovaries of two cotton cultivars, PHY499 and ST5288 under two water regimes, well-watered control and water-deficit stress grown under field conditions. All values are means ± standard error (n=4). Asterisks indicate significant difference between water regimes within the same cultivar. Cultivars within the same water regime not sharing a common letter are significantly different ($P \leq 0.05$).

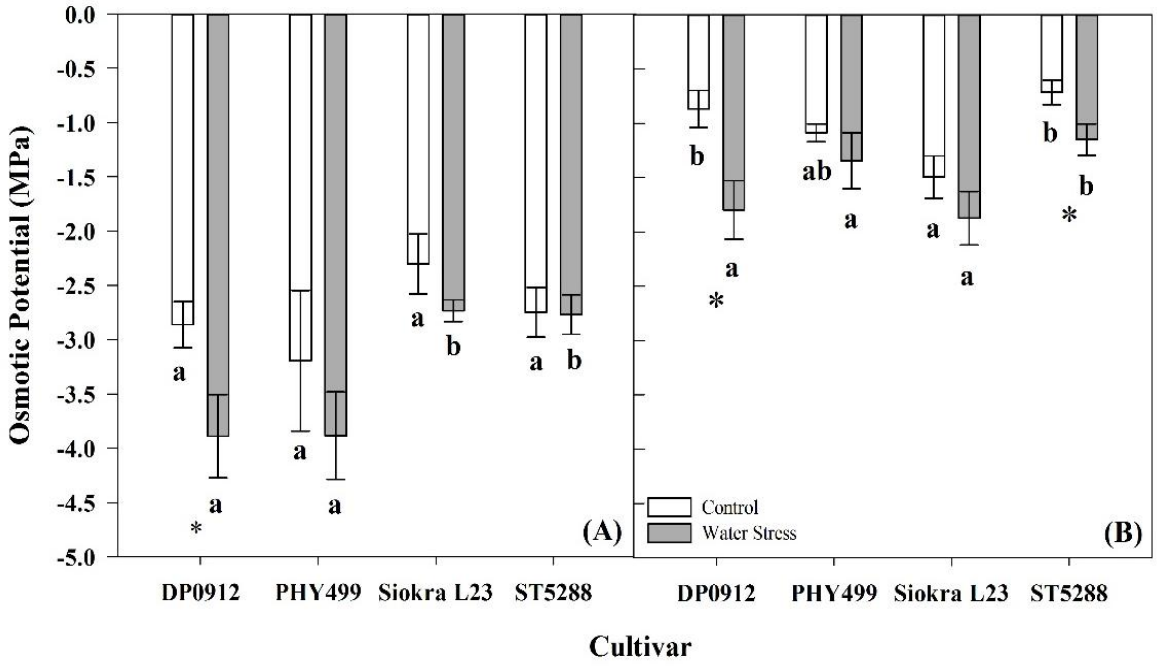

Figure 5. Osmotic potential (MPa) of four cotton cultivars, DP0912, PHY499, Siokra L23, and ST5288 under two water regimes, well-watered control and water-deficit stress in the leaves (A) and ovaries (B) of plants grown under growth room conditions. All values are means ± standard error (n=5). Asterisks indicate significant difference between water regimes within the same cultivar. Cultivars within the same water regime not sharing a common letter are significantly different ($P \leq 0.05$).

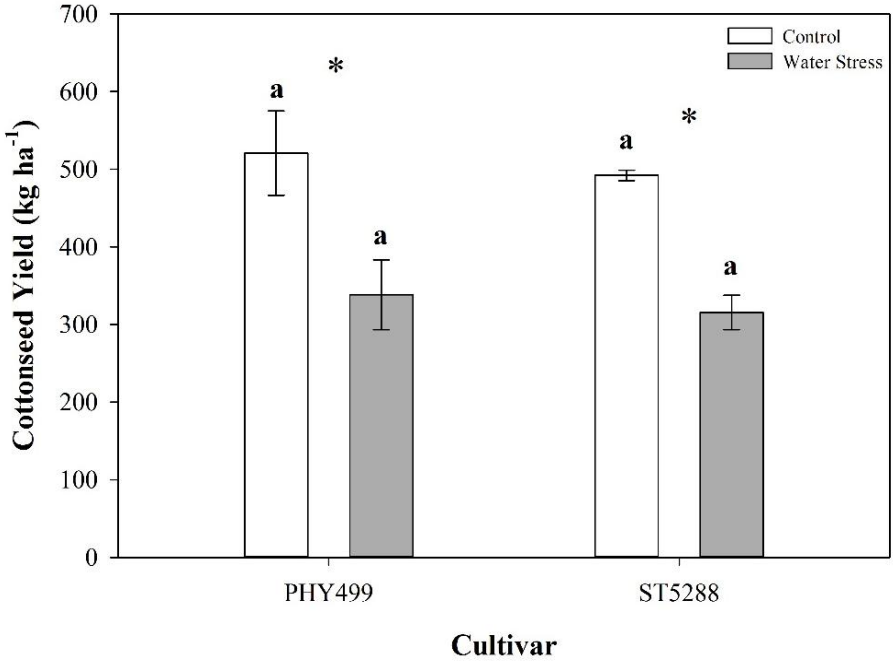

Figure 6. Cottonseed yield (kg ha^{-1}) of two cotton cultivars, PHY499 and ST5288 under two water regimes, well-watered control and water-deficit stress grown under field conditions. All values are means ± standard error (n=4). Asterisks indicate significant difference between water regimes within the same cultivar. Cultivars within the same water regime not sharing a common letter are significantly different ($P \leq 0.05$).

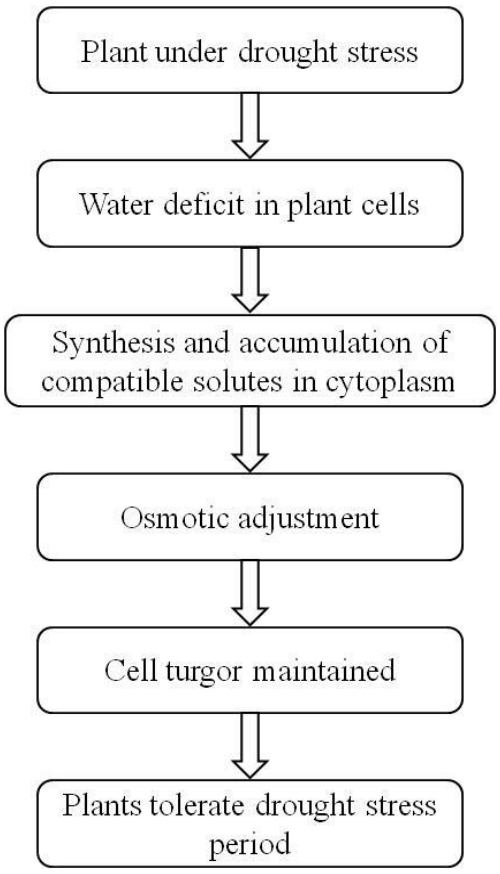

Figure 7. Flowchart of the mechanism of osmotic adjustment to tolerate drought stress in plants. (adapted from Chen and Jiang, 2010).

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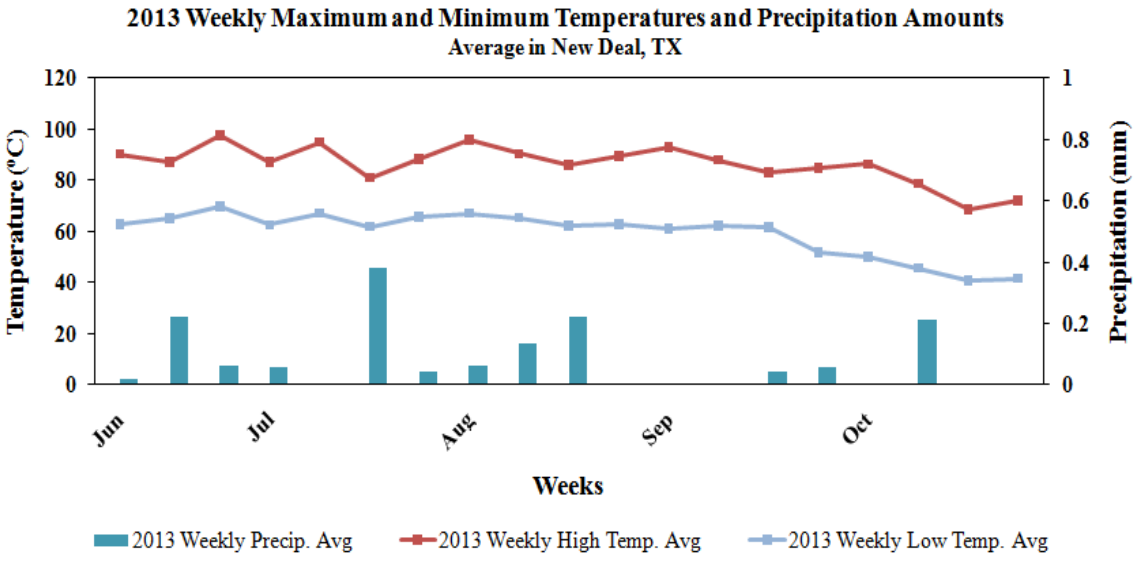
APPENDIX


2013 Weekly Maximum and Minimum Temperatures and Precipitation Amounts
Average in New Deal, TX
Temperature (°C)
Precipitation (mm)
120
100
80
60
40
20
0
1
0.8
0.6
0.4
0.2
0
Jun
Jul
Aug
Sep
Oct
Weeks
2013 Weekly Precip. Avg
2013 Weekly High Temp. Avg
2013 Weekly Low Temp. Avg

VIII. CONCLUSION

It is concluded that the physiological and metabolic processes of cotton plants were impaired by water-deficit stress during reproductive development. Leaf stomatal conductance was decreased by water-deficit stress during reproductive development of cotton plants. Photosynthetic efficiency and concentrations of pigments were also impaired by water-deficit stress at squaring stage. As a response of water-deficit conditions, activity of antioxidant enzymes was increased, with likely contribution to scavenger ROS. Carbohydrate metabolism in cotton flowers and subtending leaves was also impaired by water-deficit stress. A shift in the carbohydrate partitioning was promoted by the stress, with subtending leaves and bracts as main sources and pistils as main sinks, mainly for sucrose and soluble sugars. Additionally, proline was accumulated at high concentrations in water-stressed plants altering osmotic potential consequently inducing an osmotic adjustment. Leaves showed higher osmotic adjustment than ovaries, being considered more sensitive to water-deficit conditions. Osmotic adjustment was concluded to be a mechanism used by cotton plants to tolerate drought stress episodes.

Genotypes varied in tolerance to water-deficit stress, as more tolerant genotypes demonstrated higher osmotic adjustment in leaves and flowers. Osmotic adjustment and changes in carbohydrates metabolism could be used as effective tools in selection of drought-tolerant genotypes, and probably in plant biotechnology. As it is not likely that one single trait would be responsible for drought tolerance of plants, a combination of traits conferring tolerance to drought should be well characterized in cotton plants. Therefore, further research is needed for complete understanding of osmotic adjustment and carbohydrate metabolism in flower tissues of cotton genotypes under drought conditions during reproductive development.